UNIVERSITÀ DEGLI STUDI DI MILANO

Dipartimento di Biotecnologie Mediche e Medicina Traslazionale

SCUOLA DI DOTTORATO IN SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI

DOTTORATO DI RICERCA IN BIOTECNOLOGIE APPLICATE ALLE SCIENZE MEDICHE

XXVIII CICLO


# PLEIOTROPIC EFFECTS OF PERIFOSINE ON GLIOBLASTOMA CELLS SURVIVAL: ALTERED MEMBRANE LIPID METABOLISM AND CELL SIGNALING

Docente guida: Prof.ssa Paola VIANI

Coordinatore del Dottorato: Prof. Massimo LOCATI

Tesi di Dottorato di:

Alessandra Cinque

Matr. R10078

Anno Accademico 2014/2015

*Ai miei genitori,*
*con immenso affetto e gratitudine*

# INDEX

# ABBREVIATIONS

| | |
|---|---|
| **APLs** | Alkylphopsholipids |
| **Cer** | Ceramide |
| **CNS** | Central nervous system |
| **CT** | CTP:phosphocholine cytidyl transferase |
| **D609** | O-Tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt |
| **DMEM** | Dulbecco's Modified Eagle's Medium |
| **FCS** | Fetal Calf serum |
| **GBM** | Glioblastoma multiforme |
| **GCS** | Glucosylceramide synthase |
| **GlcCer** | Glucosylceramide |
| **GSL** | Glycosphingolipid |
| **LacCer** | Lactosylceramide |
| **LY29** | LY294002 |
| **PC** | Phosphatidylcholine |
| **PD98** | PD98059 |
| **PDMP** | D-threo1-phenyl-2-decanoylamino-3-morpholino-1-propanol |
| **PF** | Perifosine |
| **PI3K** | Phosphatidylinositol-3-OH kinase |
| **PL** | Phospholipid |
| **PPMP** | DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol |
| **SL** | Sphingolipid |
| **SM** | Sphingomyelin |
| **SMS** | Sphingomyelin synthase |
| **Sph** | Sphingosine |
| **WHO** | World Health Organization |

# SUMMARY

Glioblastoma multiforme (GBM) is the most frequent and aggressive malignant tumor of the central nervous system in adults. Despite decades of experimentation to improve the outcome of patients with GBM, this type of neoplasm remains one of the most lethal human cancers.Therefore, the need to test different and new agents for efficacy and safety is urgent. Perifosine (PF) is a synthetic lipid analogue belonging to a relatively new class of structurally related antitumor agents: the alkylphospholipids (APLs). PF exhibits potent antineoplastic activity against a multitude of cancer cell lines and different tumor models and is currently being tested in phase II clinical trial against major human tumors. However, the effect of PF against gliomas is poorly investigated. PF can induce apoptosis and/or cell growth arrest in tumor cells, but the details of its molecular mechanism is still to be elucidated. To date, the Ser/Thr kinase Akt, which is a key regulator of multiple survival pathways, is considered as the most important molecular target of PF.

However, PF can induce also Akt-independent effects and the contribution of Akt inhibition to the clinical activity of PF remains to be assessed. As other ALPs, PF may alter the structure and function of cell membranes directly by inducing a biophysical disturbance of cell membranes where it accumulates and/or indirectly by interfering with the metabolism and transport of membrane lipids. In particular, alterations in the properties of lipid rafts, ordered membrane lipid domains enriched in cholesterol and sphingolipids (SLs), could affect numerous signaling pathways crucial to cell survival and proliferation that are dependent on these structures.

On these premises, the purpose of this study was to investigate the sensitivity of GBM cells to PF treatment and to provide a contribution to the understanding of its molecular mechanism by focusing on the ability of PF to target membrane lipid metabolism and content, and, as a consequence, membrane-related signaling pathways crucial in the regulation of cell demise.

At first, we evaluated the effect of PF on cell survival in several human GBM cell lines. We demonstrated that in these cell lines PF inhibits cell viability in a dose-dependent manner and that its cytotoxic effects are not solely due to Akt inhibition. Furthermore, we found that in glioma cells PF maintains ERK in its phosphorylated/active state in a sustained manner over time. Treatment with the MAPK inhibitor PD98059 potentiates PF toxicity, and strongly reduces PF-induced LC3B-II increase. This could thus represent a molecular mechanism for self-defense from PF, at least in part due to the induction of protective autophagy. Moreover, in cells exposed to PF we found a time-dependent increase in the number of giant and multinucleated cells with an irregular shape, these morphological changes resembling those described for mitotic catastrophe, suggesting that this could be the mechanism of PF-induced cells death, while apoptosis was undetectable.

Accumulating literature indicates that in several tumor cell lines PF inhibits the rate limiting step of phosphatidylcholine (PC) synthesis, which is catalyzed by CTP:phosphocholine cytidylyltransferase, and this was associated to cell death with still unclear mechanisms. Furthermore, PC is the donor of phosphocholine in the reaction catalyzed by the enzyme sphingomyelin synthase (SMS), so the inhibition of the PC biosynthesis may also affect the sphingomyelin (SM) biosynthesis from ceramide (Cer). We found that also in GBM cells PF affects PC biosynthesis. In addition, in our model PF inhibits SM biosynthesis by affecting SMS activity, and the reduced PC seems not to represent a limiting factor for SM synthesis. The decreased utilization of Cer for SM biosynthesis results in a modest increase of Cer, which can accumulate at the endoplasmic reticulum (ER). Therefore, both the increased Cer levels, both the inhibition of PC synthesis in the ER could trigger ER stress ultimately leading to cell death. However, it is known from literature that in our GBM cells model PF fails to provoke ER stress, suggesting that other aspects of membrane lipid homeostasis, such as that involved in membrane functionality, are most probably involved in PF-induced cell death. Indeed, PC is the most abundant phospholipid in eukaryotic cellular membranes, essential for new membrane formation. On the other hand, SM together with glycosphingolipids (GSLs) represent the major SLs of the plasma membrane, displaying an asymmetric or polarized distribution, and play important roles in the regulation of membrane fluidity and sub-domain structures involved in cell signaling.

We demonstrated that PF affected the endogenous levels of phospholipids (PLs), inducing a decrease in the total PLs/protein ratio accompanied by a change in the PLs pattern. PF-treated cells were poorer in PC, SM, and phosphatidylserine (PS), and richer in phosphatidylethanolamine (PE). In addition, PF induces an about 60% increase of endogenous gangliosides content compared to untreated cells.

The dramatic changes in PLs and SLs endogenous content induced by PF could significantly affect membrane composition and functionality, which in turn can be involved in the biological response of cells to PF treatment. Indeed, we found that the inhibition of SM synthesis mimicked and potentiate PF cytotoxic effects; in addition the inhibition of gangliosides biosynthesis reverses cytotoxic action of PF thus suggesting that increase of gangliosides content is essential for its cytotoxic action.

In conclusion, PF treatment in GBM cells results in a complex network of effects where the alteration of the metabolism and content of membrane-lipid components (and maybe their related secondary messengers), seems to play a key role in determining cell death.

This study indicates that PF is decisive in its target to fight GBM cells, this representing a critical push to study new aspects in its cellular mechanisms, implying PF as anti-GBM agent.

# INTRODUCTION

## 1. HUMAN GLIOMA

### 1.1 Overview of glioma features

Central nervous system (CNS) malignancies are among the most devastating human tumors, given the central role of the brain in every aspect of bodily function (Louis et al., 2002). These cancers are often deadly, as conventional anti-tumor therapies have limited or no efficacy. The World Health Organization (WHO) recognized dozens of different types of CNS malignancies (Louis et al., 2002). CNS neoplasms present unique features compared to all the other cancers. First of all, it is harder than for other tumors to distinguish between benign and malignant lesions. Indeed, some glial tumors show benign histological features (low mitotic index, cell uniformity and slow growth), but a clinically malignant behavior, infiltrating entire regions of the brain. Furthermore, the anatomical location of the tumor can result in severe neurological impairment, e.g. when an expanding benign tumor cause brain compression in critical functional portions of the brain. (Buckner et al., 2007; Louis et al., 2002; Vescovi et al., 2006). Among the CNS tumors, gliomas are the most frequent primary brain tumors in humans, accounting for 78% of malignant CNS neoplasms. They derive from glial cells or their precursors, and very rarely metastasize outside the CNS (Buckner et al., 2007; Wen and Kesari, 2008). Based on cell line derivation, differentiation, and on their morphological appearance, gliomas are divided in: astrocytoma, oligodendroglioma, oligoastrocytoma, ependymoma, and choroid plexus tumors (Behin et al., 2003; Rich and Bigner, 2004). Among them, astrocytomas show the highest incidence (75% of all gliomas) (Dolecek et al., 2012). Astrocytomas are classified by the WHO into four prognostic grades, depending on their histological features: nuclear atypia, mitotic activity, microvascular proliferation, and necrosis (Behin et al., 2003). This grading system allows to identify, in order of increasing anaplasia, pilocytic astrocytoma (grade I), diffuse or fibrillary astrocytoma (grade II), anaplastic astrocytoma (Grade III), and glioblastoma multiforme (grade IV). Grade I and II tumors are considered with a low-grade of malignancy, while tumors belonging to the class III and IV are considered high-grade malignant gliomas (Behin et al., 2003). The IV grade astocytoma, glioblastoma multiforme (GBM), is the most frequent malignant primary brain tumor in adult and is one of the most lethal human cancers. Indeed, despite the use of the latest surgical, radiation, and chemotherapy treatments, patients with GBM have a poor prognosis, with a median survival of 12 to 15 months (Rich and Bigner, 2004; Wen and Kesari, 2008). GBMs are called “multiforme”, because these neoplasms show a high heterogenity, both interpatient and intratumor. Intrapatient heterogeneity is due to

GBM clinical presentation and its location into the brain that often prevent to completely resect this tumor. GBMs could be clinically subdivided in primary and secondary. The majority of them (> 90%) seems to arise *de novo* (primary GBMs), since they are diagnosed without evidence of a previous lower-grade tumor. They usually occur in sixty-seventy years old patients, who have a very short clinical history (less than 3 months). A smaller fraction of GBMs may arise from lower grade gliomas, with subsequent progression to higher malignancy grades (secondary GBMs). In general, secondary GBMs develop in fifty-sixty years old patients, and the time of progression from lower to higher grade lesions ranges from months to decades (Miller and Perry, 2007; Rich and Bigner, 2004). Intratumor heterogeneity is characterized by: i) macroscopic heterogeneity, with region of necrosis and hemorrhage; ii) microscopic heterogeneity, with regions showing microvascular angiogenesis, pseudopalisading necrosis, and pleomorphic nuclei and cells; iii) genetic heterogeneity, with different deletions, amplifications, and point mutations that ultimately affect multiple signal transduction and metabolic pathways; iv) cellular heterogeneity, with an highly variable cellular composition, multiple (genetically distinct) subclones, and numerous non-tumor cells that, in concert with tumor cells, create the GBM microenvironment, essential in cell-cell interactions and signaling. It is recognized that among the different cells that form GBM tumor mass exist a subpopulation of cells with stem-like properties, called GBM stem-like cells (GSCs), which are essential both in GBM initiation and aggressiveness. It has been suggested that these cells derive from transformed neural stem cells, or glial progenitor cells, that escaped the mechanisms that control proliferation and programmed differentiation. These cells are mainly localized in the subventricular zone and dentate gyrus of the hippocampus. Notwithstanding, GSCs can give rise to tumor anywhere in the brain because of their proliferative and migratory potential. Stem cells are distinct from the bulk of the tumor, but they appear to be responsible not only for tumor initiation, but also for resistance of standard treatments, recurrence, metastasis, and, at last, poor patient outcome (Dick, 2008; Merlo, 2003; Wen and Kesari, 2008). According to another hypothesis, gliomas arise from dedifferentiated astrocytes: unspecific stimulation by growth factors, e.g. transitorily released during traumatic and inflammatory conditions, may induce genetic mutations converting a mature astrocyte that retain the ability to respond to these factors into a more immature state of proliferation and migration, giving rise to gliomas (Merlo, 2003).

Notably, despite their multiple heterogeneity, different GBMs are morphologically indistinguishable and have the same clinical course, responding similarly to conventional therapy. On the other hand, targeted molecular therapies may give different results (Holland, 2000; Maher et al., 2001). GBM heterogeneity, especially regarding the existence of populations with different properties within the same tumor, is in line with the GCS model (Siebzehnrubl et al., 2011).

## 1.2 Altered signaling pathways in glioblastoma tumorigenesis

Despite the mechanisms of GBM onset and progression have not been clarified yet, a greater knowledge of the aberrations of signaling pathways associated with GBM has led to a better understanding of GBM's aggressive nature and to the discovery of new therapeutic targets (Charles et al., 2011). Indeed, despite GBMs are strikingly heterogeneous, common alterations in specific cellular signal transduction pathways and cellular functions are associated with most GBMs, and drive their aggressive behavior. Among them, there are key signaling pathways that control cellular proliferation, survival, differentiation, migration, angiogenesis, DNA repair, and apoptosis. The main signaling pathways altered in GBMs (Fig.1) include p53, tumor suppressor retinoblastoma (pRb), growth factors and growth factor receptors, PI3K (phosphatidylinositol 3-kinase)/Akt, and Ras-Raf-MAPK pathways (Furnari et al., 2007; Ichimura et al., 2004; Merlo, 2003; Rich and Bigner, 2004).

*p53 pathway*

The p53 pathway comprises the p53 protein itself, the regulatory proteins of p53, and the p53 target genes. p53 is a master regulator of the cellular response to genotoxic stresses such as UV and γ irradiation, DNA damage, defects in DNA methylation, inadequate nucleotide supply, inappropriate oncogene activation, hypoxia, and cytotoxic drugs. In these stressful conditions, p53, that is a short-lived transcription factor, is upregulated and acts promoting the expression of both proteins that halt the cell cycle and proteins involved in DNA repair. If the amount of damage is beyond the cell's capacity for repair, p53 facilitates the expression of proteins involved in apoptosis, inducing cell death. So, the p53 tumor suppressor prevents the propagation of cells with an impaired genome, a consequence of which might be tumor formation. Indeed, a deficient p53 pathway functioning leads to a greater tolerance of genomic instability, possibly resulting in widespread genomic alterations, which are frequently found in many cancer, among which malignant glioma. Loss of normal p53 function has been linked to gliomagenesis. This hypothesis is strengthened by the increased incidence of gliomas in patients with Li-Fraumeni syndrome, a familial cancer-predisposition syndrome associated with germline p53 mutations. TP53 (the gene encoding p53) inactivating mutation or loss is frequent in childhood gliomas, low-grade gliomas, anaplastic astrocytomas, and secondary GBMs. On the other hand, mutations or amplifications in regulators of p53 levels and activity are more common in primary GBM. Indeed, the chromosomal region containing Human doubleminute 2 (HDM2; the human xenologue of mouse double-minute 2 MDM2), that is one of

p53 downstream gene targets and, maybe, its most important modulator, is amplified in about 10% of primary GBM. HDM2 inhibits p53 transcriptional activity by binding to the p53-transactivating domain, inducing nuclear export, or functions as an E3 ubiquitin ligase, facilitating ubiquitin-mediated p53 degradation by proteasome. Furthermore, another p53 downstream gene target encoding $p14^{ARF}$ protein, that negatively regulates HDM2 ability to target p53 for degradation, is often deleted or methylated in malignant gliomas (Ichimura et al., 2004; Merlo, 2003).

*pRb pathway*

The retinoblastoma protein (pRb) is a major regulator of cell-cycle progression, regulating G1/S transition. In quiescent cells, pRB is in its hypophosphorylated state and blocks proliferation by binding and sequestering the E2F family of transcription factors, and thus preventing the transactivation of genes essential for progression through the G1/S restriction point. Under mitogenic stimulation, the MAPK cascade is activated and promotes the expression of CyclinDs (D1-D3) and their association with the cyclin-dependent kinases (CDK)4 or 6, as well as the degradation of the CDK2/CyclinE inhibitor $p27^{Kip1}$. These activated Cyclin-CDK complexes phosphorylate pRb, causing release of sequestered E2F transcription factors, that in turn transactivate the genes required for cell cycle progression into S phase and for DNA synthesis. The formation of the Cyclin-CDK complexes is negatively regulated by CDK inhibitors (CKIs) $p16^{INK4A}$ and $p15^{INK4B}$, which act by inhibiting the binding of CyclinDs to CDK4/6 and by displacing $p27^{Kip1}$, which in turn binds and inhibits CDK2. In gliomas unscheduled cell-cycle entry, that favor unrestricted cellular proliferation, is due to any of the following several genetic alterations: mutational inactivation of Rb1 (the gene encoding for pRb) itself or the CKIs, epigenetic down-regulation of CKIs levels, or amplification/overexpression of CDKs4/6 or of the CyclinDs. Alteration of pRB pathway effectors or inhibitors occurs in both primary and secondary GBMs in a mutually exclusive manner. In low-grade gliomas mutations of cell-cycle components are uncommon, but the transformation from low-grade to intermediate-grade glioma is frequently related to dysregulation of the pRb pathway, highlighting the importance of its inactivation in glioma progression (Ichimura et al., 2004; Merlo, 2003).

*Growth factor signaling*

Inappropriately active cellular proliferation is a typical feature of most advanced cancers, including malignant gliomas. The disruption of the pRb pathway abolishes restrictions to proliferation, but

growth factor pathways give the proliferative stimulus in most GBMs. In addition to cellular proliferation, growth-factor pathways regulate many other pro-tumorigenic cellular functions such as apoptotic resistance, motility, invasion, and neo-angiogenesis. Numerous growth factor pathways induce the GBM phenotype, including the pathways of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF1), vascular endothelial growth factor (VEGF), and transforming growth factor-ß (TGF-ß). Specific molecular constituents define each growth factor pathway, but the activation mechanism and the phenotypic impact of the pathways in GBMs are similar. Usually, growth factor ligands bind specific cell-surface receptors, forming a signaling complex, inducing receptor phosphorylation and activation. After phosphorylation, the receptors undergo conformational changes that allow them to bind intracellular effectors in order to propagate the pathway signal. To halt growth-factor pathway activity, the receptors are rapidly inactivated, internalized and degraded. There are different mechanisms by which gliomas may activate receptor pathways: i) overexpression of ligand and/or receptor, leading to an autocrine loop when both ligands and receptors are overexpressed; ii) mutations of receptor, leading to its constitutive activity in the absence of ligand or to its increased response to ligand because of its altered stability; iii) mutations and/or altered expression of genes coding for intracellular messengers or for their regulators, leading to intracellular messengers activation (Miller and Perry, 2007).

*PI3K /Akt pathway*

The PI3K/Akt signaling pathway is frequently altered in GBMs, due to oncogenic alterations in key elements of the pathway or aberrations in its regulation. PI3Ks are a family of protein and lipid kinases. Different classes of PI3Ks have been identified. Among them, the class $I_A$ PI3K, which is activated by receptor tyrosine kinases (RTKs) and also by the small GTPase Ras, seems to play an important role in tumorigenesis. Class I PI3Ks are able to catalyze the phosphorylation of phosphoinositol (PI) on the inner leaflet of cell membrane, and in particular of phosphatidylinositol-4,5-bisphosphate ($PIP_2$), which is converted in phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$). $PIP_3$ acts as a second messenger and recruits the serine/threonine kinase Akt to the plasma membrane by binding to its pleckistrin homology (PH) domain. Membrane translocation induces Akt conformational changes enabling its activation through phosphorylation at the two amino acid residues T308 and S473 by phosphoinositide-dependent kinase (PDK) 1 and PDK 2, respectively. Once activated, Akt phosphorylates numerous targets starting a series of signaling cascades involved in diverse cellular functions. The PI3K/Akt pathway positively regulates survival

pathways, promotes cell proliferation, regulates lipids and glucose metabolisms, cell movements and vesicle trafficking. The PI3K/Akt pathway is negatively regulated by the lipid phosphatase PTEN, that converts $PIP_3$ back to $PIP_2$, counteracting PI3K activity. The PI3K/Akt pathway is one of the most frequently hyperactivated signaling pathways in GBM. Alterations in this pathway include overexpression and mutations of RTKs genes, inactivating mutations or deletions of PTEN tumor suppressor gene, and oncogenic mutations in ras genes. Aberrant activation of PI3K/Akt pathway results in uncontrolled cell growth, resistance to cytotoxic treatments, massive angiogenesis, and migration/invasiveness (Chakravarti et al., 2004; Furnari et al., 2007; Holand et al., 2011; Rich and Bigner, 2004).

*The Ras-Raf-MAPK pathway*

The MAPK pathway is a signaling cascade, evolutionarily conserved, that consist of a set of protein kinases that ultimately transduces signals from the cell membrane to the inner of the cell and modulates gene expression. The MAPK pathway plays a pivotal role in regulating many biological functions, such as cell proliferation, migration, survival, and differentiation.

This pathway is constitutively active in many tumors. Importantly, MAPK pathway is altered in 88% of adult GBMs. The Ras-Raf-MEK-ERK signaling pathway is hyperactive in GBMs owing to an inappropriate activation of its upstream regulators, such as RTKs and/or integrins.

On the other hand, unlike most cancers, somatic mutations of the RAS and BRAF (a RAF kinase) genes are very rare in malignant gliomas. However, altered Ras-Raf pathway, beyond the somatic mutations, plays an important role in the GBMs biology. Indeed, all three RAF proteins (ARAF, BRAF, and RAF1) are frequently overexpressed in human malignant gliomas. In addition, it has been reported that gliomas carry extra copies of the wild-type BRAF gene and that a significant correlation exists between activated MAPK and BRAF copy number gains. It was also found that this gene copy number gain is prevalent in high-grade gliomas.

Of relevance, genetic and tissue-specific gene transfer studies demonstrate that constraining normal astrocytes and neuronal progenitors to express oncogenic RAS and BRAF leads these type of cells to shift into malignant gliomas, showing a crucial role of Ras-Raf pathway alterations in gliomagenesis. The abnormal activation of this pathway may also have a role in the sustainment of GBMs. Indeed, inhibition of Ras-Raf-MAPK pathway with different mechanisms (e.g. through transcriptional down-regulation and small molecular inhibitors) reduces glioma growth (Lo, 2010).

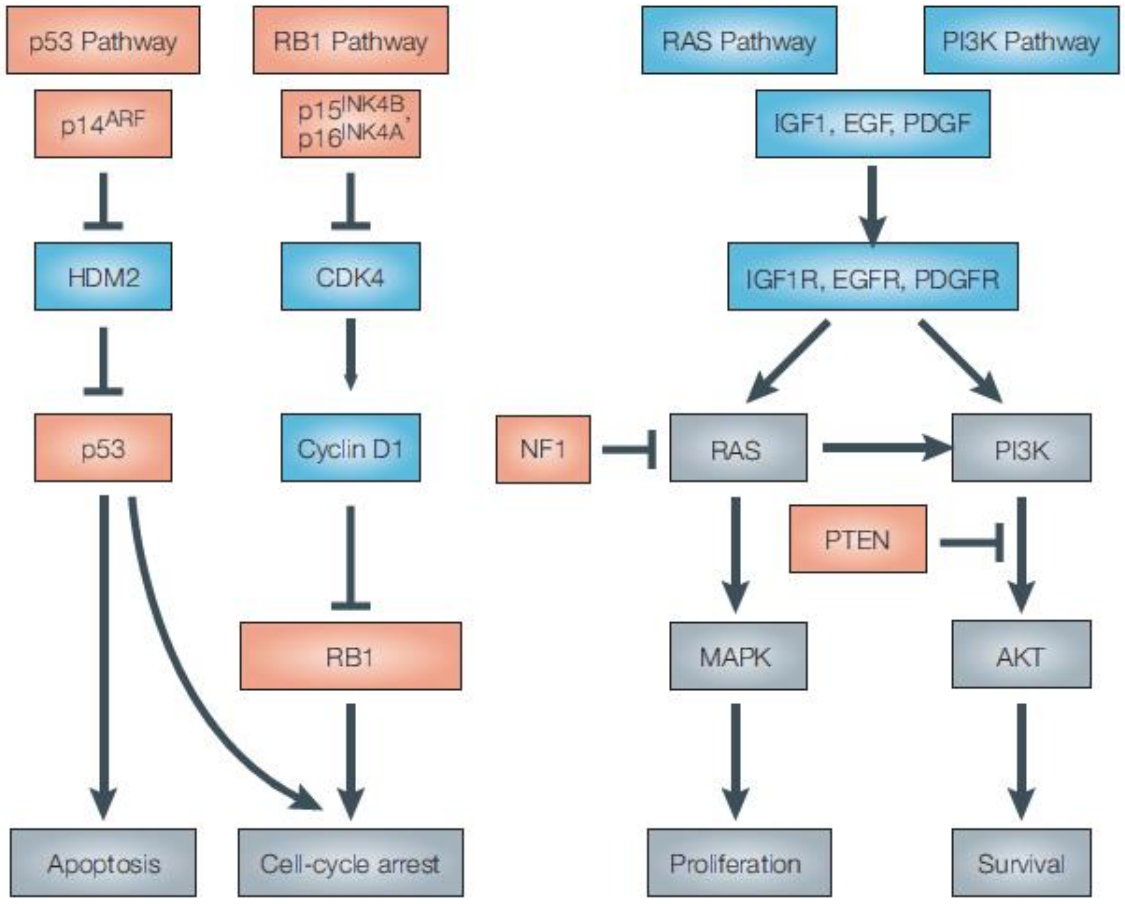


**Figure 1.** *Cellular pathways frequently altered in gliomas* (Rich and Bigner, 2004).

## 1.3 Glioblastoma treatment

Despite the intense efforts of scientists, neurologists and neurosurgeons to develop effective therapeutic approaches to defeat GBM, this tumor is still among the most deadly types of human tumors (Clarke et al., 2010; Rich and Bigner, 2004). The GBMs are so difficult to treat mostly because of their location and their complex and heterogeneous biology (Holland, 2000; Mrugala, 2013). One of the major challenges in therapy of these type of tumors is associated with brain parenchyma vulnerability. Indeed, intracranial tumors exhibit a complex interaction with normal brain parenchyma, which enormously complicates the use of conventional therapies and the development of new treatments, since even minimal disturbance to the neural cytoarchitecture or circuitry can lead to dramatic functional disturbances (Holland, 2000; Louis et al., 2002; Vescovi et al., 2006). Another obstacle for the treatment of GBM is the presence of the blood-brain barrier (BBB), which hinders most therapeutic drugs to reach tumor mass in adequate concentrations (de Vries et al., 2006). The current therapeutic strategies for GBMs consist in maximal surgical resection, when achievable, followed by radiotherapy and chemotherapy (Clarke et al., 2010;

Holland, 2000). Unfortunately, GBMs are characterized by high levels of cellular proliferation and diffuse invasion into neighboring brain structures, hindering total surgical extirpation of the tumor, which inevitably recurs (Holland, 2000; Mrugala, 2013). Specialized techniques such as intraoperative MRI (magnetic resonance imaging), MRI-guided neuronavigation, functional MRI (Asthagiri et al., 2007), fluorescence-guided surgery (Stummer et al., 2006), awake craniotomy, and electrocortical mapping have enhanced surgery safety and allowed greater surgical resection of the tumor mass. Moreover, surgical debulking alleviates mass effect-symptoms, furnishes tissue for histological diagnosis and molecular studies, and enables to safely administrate subsequent treatments, such as radiotherapy and/or chemotherapy (Buckner et al., 2007), that slightly ameliorate patient prognosis. For example, it has been shown that radiotherapy treatment increases patients survival from 3-4 months to 7-12 months (Walker et al., 1978). In this regard, innovative radiation treatments have been designed using multiple field techniques to deliver high doses of radiation to tumor, but not to normal brain tissue, therefore hopefully decreasing the damage caused by radiations in the healthy areas. More in detail, these new radiation-based approaches are: the fractionated radiotherapy, that exploit the differing radiosensitivities of normal tissues and neoplasms, the stereotactic radiosurgery, that deliver a high radiation dose in a single fraction to an image-defined target, and stereotactic radiotherapy, that combines fractionated radiotherapy to stereotactic radiosurgery (Buckner et al., 2007). As previously mentioned, the surgical resection and the radiotherapy are often accompanied to chemotherapy, that may enhance the effectiveness of surgery and radiation approaches, improving GBM patient survival (from 6 to 10% increase in the 1-year survival rate) (Buckner et al., 2007; Fine et al., 1993; Stewart, 2002). However, many limitations are associated with chemotherapy, as chemotherapeutics' ability to cross the BBB, determined by size and lipophilicity of molecules, integrity of BBB, and presence of an active efflux pumps. GBMs impair the BBB integrity both structurally and functionally, but not in a uniform manner. The loss of the BBB in general does not occur along the infiltrating edges of the tumor, so that chemotherapy drugs do not reach the entire tumor at an effective concentration (de Vries et al., 2006; Jain et al., 2007; Neuwelt et al., 2011). Different strategies exist to overcome this drug delivery problem: increase the dosage of some drugs, conjugate drugs to lipophilic moieties or other vectors, package drugs in carrier systems (liposomes, micelles and dendrimers), co-administrate drugs with inhibitors of BBB drug-efflux transporters, and infuse drugs directly into the tumor by a catheter or implanted therapies consisting of therapy-infused reservoirs or matrices (convection enhanced delivery) (Laquintana et al., 2009).

The most commonly used chemotherapy drug in the treatment of high-grade malignant gliomas is temozolomide, an oral alkylating agent with good penetration of the blood-brain barrier, which has

showed therapeutic benefit (Buckner et al., 2007). However, the outcome of the standard treatment remains poor. Therefore, a hard work has been made in the last decades to identify new therapeutic targets to develop effective strategies to treat this so devastating and deadly cancer. These novel promising approaches include: gene therapy, in order to transfer lethal genes to the tumor cells; the use of viruses able to infect the tumor cells and then kill them lytically; and therapies that entice the immune system to reject the tumor (Holland, 2000). In addition, in recent years it is emerging the awareness that a multi-targeted therapies are essential to treat and defeat GBM, due to its extremely complex and heterogeneous molecular biology. For this reason, the need to test different and new agents for efficacy and safety is urgent (Mrugala, 2013).

## 2. SPHINGOLIPIDS

### 2.1 An overview of sphingolipids

Sphingolipids (SLs) were discovered more than a century ago by J. L. W. Thudichum, while studying the chemical composition of the brain. He coined the term "sphingosine" (hence the root term "sphingo-") to indicate the backbone of all SLs, based on the Greek mythological Sphinx, because of its enigmatic properties (van Echten-Deckert and Herget, 2006). SLs are ubiquitous and essential structural components of eukaryotic membranes. However, in the last 20 years, advances in biochemical and molecular research highlight a pivotal role of SLs as bioactive metabolites in regulating a wide range of crucial cellular processes such as cell growth, death, adhesion, migration, and senescence (Hannun and Obeid, 2008). SLs have also been reported to dynamically cluster with sterols to form lipid microdomains (membrane rafts), which have been suggested as critical platforms for cell signaling (Simons and Ikonen, 1997). Concerning their chemical structures, SLs are amphipatic molecules composed by a hydrophobic moiety (ceramide, Cer), that acts as membrane anchor, and a hydrophilic headgroup. Cer, the structural element common to all SLs, is constituted by a long-chain amino alcohol (sphingoid base), connected by an amide linkage to a fatty acid, generally with a long-chain (palmitic (C-16) or stearic (C-18) acid or longer, sometimes hydroxylated). The most common sphingoid base in humans is sphingosine (Sph), containing 18-20 carbon atoms (C-18 or C-20) and a C4-C5 double bound in the *trans*-D-erythro conformation, which is essential for some of the bioactive roles in which Sph-based SLs are involved. A number of other sphingoid long-chain bases exist, e.g. C-18 and C-20 sphinganines, which lack the double bond, the less common phytosphingosine, which carries a hydroxyl group on C-4, and methylsphingosine with a methyl group at C-15 (Lahiri and Futerman, 2007; Tettamanti et al., 2003). Cer is the central building block of all complex SLs, which are synthesized by addition of

polar molecules (representing the polar headgroup) to its primary alcoholic residue (Lahiri and Futerman, 2007). A large variety of polar headgroups could be attached to Cer, including charged, neutral, phosphorylated, or glycosylated moieties. For example, attachment of phosphocholine forms sphingomyelin (SM), whereas attachment of glucose or galactose leads to the generation of glucosylceramide (GlcCer) and galactosylceramide (GalCer) respectively, used as precursors for the synthesis of more complex glycosphingolipids (GSLs). GSLs are the most structurally diverse class of complex SLs, and are normally classified as acidic or neutral. The neutral GSLs include GlcCer and GalCer (cerebrosides), while acidic GSLs carry an oligosaccharide chain containing one or more sialic acid residues (gangliosides), sulphate moieties (sulphatides) or glucuronic acid. The oligosaccharide moiety of both neutral and acidic GSLs may contain up to 15-20 saccharidic units (rarely more), with the main sugars being glucose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine. In addition, an attachment of phosphate at the carbon in position 1 (C-1) of the sphingoid base (Sph) or Cer forms Sph-1-phosphate (S1P) and Cer-1-phosphate (C1P), respectively (Lahiri and Futerman, 2007; Tettamanti et al., 2003). Therefore, the complexity of SLs is based on three structural constituents: the sphingoid base, the fatty acid, and the head moiety, keeping in mind that at least five different sphingoid bases have been identified in mammalian cells, 20 species or more of fatty acid (differing in chain length, degree of saturation, and hydroxylation grade) can be attached to the sphingoid base, and more than 500 different saccharides structures have been characterized in GSLs. Despite some preference of specific components to associate in particular SLs (e.g., certain fatty acids are frequently found in certain GSLs), it is obvious that the number of potential combinations is nonetheless disconcerting. The reason for such a variety of SLs structures is not known. It is still unclear if each distinct SL or GSL structure has its own unique role or rather the combinatorial pattern of SLs and GSLs at any one time and their distribution (or segregation) over the plasma membrane surface define their roles. However, such complexity demands highly organized and complex mechanisms of regulation at both the biochemical and cellular levels, that for the most part are still to be clarified (Futerman and Hannun, 2004; Lahiri and Futerman, 2007).

## 2.2 Sphingolipids and membrane lateral organization

The bulk structure of biological membranes consists in a bilayer of amphipathic lipids. The creation of the lipid bilayer is a first level of ordered organization of the membrane and is a consequence of the aggregational properties of complex amphipathic membrane lipids (Sonnino and Prinetti, 2013). The fluid mosaic model for cellular membrane structure, proposed by Singer and Nicholson, introduced the concept that the glycerophospholipid bilayer is a two-dimensional fluid construct

that allows the lateral movement of membrane components (Singer and Nicolson, 1972). During the past decades, it has become clear that lipids are not only the building blocks contributing to the membrane basic organization, but they may play active roles in modulating specific membrane proteins such as receptors, carriers, pumps, and enzymes. Moreover, some of the lipid components undergo lateral phase separation and influence the membrane curvature, asymmetry and strain within the two lipids layers, creating therefore multiple levels of lateral order able to form membrane microdomains endowed by different compositions and functions (Sonnino and Prinetti, 2010). In the late 1980s, a growing number of experimental findings collectively gave rise to the lipid domain hypothesis. The notion that different levels of order exist in biological membranes is now deeply rooted in cellular biology. Lipid domains are commonly defined as fluctuating nanoscale assemblies of SLs, cholesterol, and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking and that consequently are involved in a great variety of cellular functions and biological events (Lingwood and Simons, 2010). SLs are ubiquitous membrane components, being present in different organelle membranes and particularly abundant in the plasma membrane, where SM and GSLs represent the major SLs (Hakomori, 1990). SLs are amphipathic molecules, which accounts for their tendency to aggregate into membranous structures, with an hydrophobic portion (constituted by Cer) embedded in the lipid core of biological membranes and a hydrophilic portion protruding in the extracellular milieu (Feizi, 1985). Differently from glycerolipids, SLs, and GSLs in particular, exhibit molecular structure and conformational properties which give them a strong tendency to segregate within phospholipid bilayers, forming subsequently laterally separated phases characterized by reduced fluidity and hydrocarbon chain mobility (Sonnino and Prinetti, 2010; Tettamanti et al., 2003). Indeed, the Cer backbone of SLs is a relatively rigid system and is characterized by the presence of the amide nitrogen, the carbonyl oxygen and the hydroxyl group positioned in proximity of the water/lipid interface of the bilayer, which confers to SLs to act both as donors and acceptors for the formation of hydrogen bonds (Pascher, 1976). The formation of a network of hydrogen bond at the water/lipid interface of the bilayer considerably stabilizes the SLs segregation in specific membrane areas: the lipid membrane domains, also defined as "SL-rich membrane domains" (Pascher, 1976).

Furthermore, in the case of GSLs, the presence of the bulky oligosaccharide hydrophilic headgroup strongly favor phase separation and spontaneous membrane curvature, due to the geometrical properties of different GSL headgroups (Sonnino and Prinetti, 2010). It has also been proposed that phase separation of GSLs could be favored by the formation of carbohydrate-carbohydrate interactions, i.e. hydrogen bonds involving the GSL sugar head groups, even if data proving this are lacking (Brocca et al., 1998). However, the hydrophylic character of sugars and the necessity to

avoid repulsion between the negative charged oligosaccharide attract water, which could have a role in organizing a net of hydrogen bonds able to stabilized GSL clustering (Bach et al., 1982; Brocca et al., 1998; Cantu et al., 1990; Ha et al., 1989; Heatley and Scott, 1988). Moreover, membrane complex lipids, such as glycerophospholipids, are characterized by a highly heterogeneity in their fatty acid composition, such as bearing acyl chains with different number of carbon atoms and unsaturations (Sonnino and Prinetti, 2010). This high degree of unsaturation provides membrane fluidity, a characteristic that is fundamental for the functional properties of a biological membrane. Lipids, such as SLs, mostly characterized by the presence of long saturated acyl chains (that can be tightly packed in the hydrophobic core of a bilayer), have the tendency to segregate within phospholipid bilayers into more ordered phases (Prinetti et al., 2009). It was estimated that distribution in the fluid phase of a phospholipid bilayer is inversely correlated with the acyl chain length and directly correlated with the degree of unsaturation (Palestini et al., 1995). Notably, SLs display an asymmetric distribution in biological membranes, being present essentially in the outer monolayer. This raises the question: how could ordered domains form in the inner leaflet? To answer this question, it was suggested that interdigitation of long chain fatty acid residues of complex membrane lipids might be responsible for the tethering of the internal leaflet to the external one, influencing events localized on the cytosolic side of the membrane (Chiantia and London, 2013). As mentioned before, segregation of membrane SLs is responsible for the creation of less fluid membrane regions, where membrane-associated proteins can be confined. Several classes of membrane-associated proteins display a strong preference for the association of lipid-rich membrane domains, e.g. glycanphosphoinositide-GPI-anchored proteins, or protein with a lipid modification such as myristoylation/palmitoylation and double palmitoylation, which target proteins to lipid domains. Transmembrane proteins are, in some cases, also concentrated in lipid rafts. Finally, peripheral proteins can be associated or recruited to lipid rafts, possibly indirectly via interactions with raft-resident proteins (Brown, 2006; Prinetti et al., 2009). The association with lipid domains can affect the functional properties of a membrane protein in different ways: (a) the association with lipid domains could represent a mechanism to facilitate the co-clustering of different membrane proteins or, conversely, the trapping of a protein within lipid rafts could prevent it from interacting with other proteins preferentially localized in fluid membrane regions; (b) the association of a protein with a rigid membrane zone could induce protein conformational changes affecting its functional activity; (c) proteins confined in lipid domains have a higher probability to laterally interact with specific lipids that can modulate their activity. For example, it has been amply demonstrated that GSLs (in particular, gangliosides) can interact and modulate the activity of plasma membrane proteins, such as receptor tyrosine kinase, even if in most cases it is still unclear

the molecular aspects of GSL-protein interactions, underlying the modulatory effect of GSLs (Prinetti et al., 2009). The oligosaccharide chain of a GSL inserted in the plasma membrane could interact with a membrane protein via a) amino acid residues belonging to the extracellular loops of the protein, if the conformation of the polypeptide chain allows them to be sufficiently close to the membrane surface; b) sugar residues in the glycans of a glycosylated protein, if the dynamics of the protein oligosaccharide chain allows the correct orientation toward the cell surface; c) the hydrophilic portion to the anchor in the case of GPI-anchored proteins (that is surely located in proximity of the extracellular surface of the membrane) (Prinetti et al., 2009).

Notably, GSLs present an oligosaccharide chain directly involved in processes of cell social behavior. Indeed, the hydrophilic component is able to provide recognition sites for external ligands/agents/cells, eliciting a series of molecular events controlling fundamental cell functions such as proliferation/arrest of proliferation, differentiation/apoptosis, embryogenesis, ageing.

The list of receptor functions of GSLs is extensive. For example, gangliosides can act as toxin receptors: cholera toxin from Vibrio cholera, and Escherichia coli heat-labile enterotoxin bind to GM1 (Angstrom et al., 1994), and Shiga toxin from Shigella dysenteriae binds to Gb3 (Keusch et al., 1991). GSLs also mediate E selectin-dependent rolling and tethering (Schnaar, 2004), 9-O-acetyl GD3 plays a role in neuronal motility (Mendez-Otero and Cavalcante, 2003), and a-GalCer acts as a ligand recognized by a special group of immune T cells, known as invariant NKT cells (Hayakawa et al., 2004). Cer is present in small amounts within cell membranes, as it functions primarily as an intermediate of complex SL metabolism and acts as a cell signaling mediator (Futerman and Hannun, 2004). However, in some circumstances, it plays an active role in membrane lateral organization and dynamics (Goni and Alonso, 2006, 2009; Staneva et al., 2008). For example, Cer can induce the coalescence of large plasma membrane domains, promoting the raft residing CD95/Fas death receptor clustering, and subsequently inducing apoptosis (Grassme et al., 2003). The increase of Cer at the membrane domain level can be relevant to different physiological processes. It can be induced, for example, by the action of signaling-related sphingomyelinases (SMases), contributing to the definition of signaling platforms (Staneva et al., 2008). Furthermore, Cer formation may induce a displacement of cholesterol from membrane domains, affecting membrane cholesterol homeostasis and so the activity of cholesterol-bound proteins (Goni and Alonso, 2009; Staneva et al., 2008). Indeed, SLs have a restricted ability to shield the small polar groups of Cer and cholesterol from contact with water and so Cer and cholesterol compete in their association with lipid domains (Goni and Alonso, 2009; Staneva et al., 2008). Finally, the formation of Cer-enriched microdomains can promote membrane vesiculation and vesicle budding. The Cer-mediated vesicles budding could have a role in the sorting of

subcellular membranes into different populations of intracellular vesicles. It has also been implicated in the ER-Golgi vesicular traffic of both SLs and proteins (Giussani et al., 2006; Trajkovic et al., 2008).

## 2.3 Sphingolipid metabolism

Common synthetic and catabolic pathways govern the metabolism and catabolism of SLs, regardless of the heterogeneity in their structures and functions (Adan-Gokbulut et al., 2013).
Pathways of SL metabolism have a unique metabolic entry point, constituted by the reaction catalyzed by serine palmitoyl transferase (SPT), which forms the first SL in the de novo pathway, and a unique exit point, constituted by the reaction catalyzed by S1P lyase, which breaks down S1P into non-SL molecules. The numerous metabolic steps form a highly intricate network, connecting the metabolism of many SLs. In this network, Cer occupies a central position in SLs biosynthesis and catabolism (Hannun and Obeid, 2008). SLs can originate from both the *de novo* pathway (Fig.2) or through the hydrolysis of complex SLs. The first reactions of the *de novo* biosynthetic pathway are catalyzed by membrane-bound enzymes active at the cytosolic face of the endoplasmic reticulum (ER), leading to the formation of Cer. The *de novo* pathway begins with the condensation of the amino acid L-serine with palmitoyl-CoA (or, less frequently, stearoyl-CoA), catalyzed by the enzyme serine-palmitoyl (or -stearoyl) transferase, to give rise to 3-keto-dihydrosphingosine, which is subsequently reduced by a NADPH ($H^+$)-dependent oxidoreductase, 3-ketosphinganine reductase, to produce dihydrosphingosine (sphinganine). Dihydroceramide synthase (sphinganine N-acyl transferase) next acylates dihydrosphingosine to form dihydroceramide (dhCer), using, as acyl donor, fatty acyl-CoAs. Dihydroceramide synthase is also able to catalyse the acylation of sphingosine (Gault et al., 2010). In mammalian cells, six different isoforms of dhCer synthase (dhCerS/CerS) have recently been identified, and are encoded by six different genes, formerly known as longevity assurance (LASS) genes. The proteins encoded by these genes show substrate preference for specific chain length fatty acyl CoAs and so are responsible for the fatty acid composition of Cer. For example, CerS1 is involved in the production of C-18Cer, while CerS5 and CerS6 preferentially catalyze the acylation of (dh)Sph with myristoyl-, palmitoyl-, and stearoyl-CoA compared with very long-chain fatty acyl-CoAs. These enzymes share common biochemical features, such as their catalytic mechanism, their structure, and intracellular localization, whereas show unique tissue distribution and different biological properties. Indeed, they are differently involved in a wide range of processes as diverse as cancer and tumor suppression, in the response to chemotherapeutic drugs, in apoptosis, and in neurodegenerative diseases (Levy and Futerman,

2010; Pewzner-Jung et al., 2006). The major part of the dhCer pool is then desaturated at the 4,5 position of the sphingoid base tail, by the action of dhCer desaturase (that uses $NADPH^+$ $H^+$ and $O_2$), with formation of Cer (Michel and van Echten-Deckert, 1997). The neo-synthesized Cer reaches directly the PM or is used as common precursor for SM and GSLs biosynthesis. Intriguingly, despite the large number of known GSL structures, only glucose and galactose have been found to be directly attached to Cer leading to GlcCer and GalCer, respectively. GalCer is synthesized from Cer and UDP-galactose by the action of Cer galactosyltransferase (CerGalT), an enzyme that is expressed mostly in oligodendrocytes and Schwann cells, but also in neurons and astrocytes. CerGalT is localized in the ER with a luminal orientation of its catalytic site, so Cer should be translocated from the cytosolic side, where it is formed, to the luminal side of ER membrane to be galactosylated. GalCer is then transferred to the lumenal Golgi compartment, where it is submitted to either sulphation to form sulfatide, sialylation to form ganglioside GM4, or further galactosylation to form $Gal_2Cer$, the precursor for a rather restricted number of gala-series GSLs. GalCer is most abundant in oligodendrocytes and Schwann cells. GalCer and its sulphated derivative sulfatide represent 30% of the total lipid content of myelin (the specialized plasma membranes of Schwann cells in the PNS and oligodendrocytes in the CNS) and are essential to confer rigidity and stability to the membranes, allowing a correct conduction of nerve impulses (van Echten-Deckert and Herget, 2006). GlcCer biosynthesis occurs on the cytosolic leaflet of the Golgi apparatus, from Cer and UDP-glucose, through a reaction catalyzed by GlcCer synthase (GCS) (Jeckel et al., 1992). Whereas a minor part of the neo-synthesized GlcCer can directly reach the plasma membrane (probably via vesicular system), most of the GlcCer is translocated by a flippase enzyme to the luminal side of the cis-Golgi compartment, where further glycosylations take place. The first glycosylation, catalysed by lactosyl-Cer synthase is galactosylation of GlcCer to lactosylceramide (LacCer), which is the common precursor for all GSL-series, except the gala-family (see above). LacCer is sialosylated to GM3, GM3 to GD3, and GD3 to GT3, by the action of three sialyltransferases (SAT I, SAT II and SAT III), each recognizing specifically the acceptor substrate. GM3, GD3 and GT3, are the starting points for the "a-series", "b-series" and "c-series" gangliosides, respectively. Along each series, non specific N-acetyl-galactosaminyltransferase, galactosyl-transferase and sialyl-transferase (SAT IV) introduce in sequence a residue of N-acetylgalactosamine, galactose, and sialic acid, respectively, giving rise to more complex gangliosides. Further sialosylations can be accomplished by sialosyl-transferase V (SAT V). From LacCer a further series of GSLs ("o-series") can originate from the sequential action of N-acetyl-galactosaminyl-transferase, galactosyl-transferase and sialyl-transferase IV and V, producing GA2 (asialo-GM2), GA1 (asialo-GM1), and gangliosides GM1b, GD1c and $GD1_\alpha$ (Degroote et al., 2004;

Ichikawa and Hirabayashi, 1998; Tettamanti et al., 2003; van Echten-Deckert and Herget, 2006). Gangliosides are major components of neuronal membranes, where they constitute 10–12% of the total lipid content (20–25% in the outer membrane layer) (Tettamanti et al., 2003). As regard to SM, its biosynthesis occurs by transferring a phosphocholine headgroup from phosphatidylcholine to the primary alcoholic residue of Cer, through the action of SM synthases (SMS), thereby also generating diacylglycerol. SM represents approximately 10% of the lipids present in mammalian cells. Until now, two different enzymatic SMS isoforms have been identified: SMS1, localized in the luminal side of the cis/medial Golgi apparatus and SMS2, primarily localized to the plasma membrane (Hannun and Obeid, 2008; Tafesse et al., 2006). Experimental evidence revealed that the most of the *de novo* synthesis of SM (~ 90%) occurs in the Cis/medial Golgi, while only a minor part occurs at the plasma membrane level. It was suggested that SMS2, being localized at the plasma membrane, plays a role as regulator of SM and Cer levels for signalling pathways and signal transduction (Giussani et al., 2014).

As Cer synthesis occurs in the ER and that of GlucCer, complex GSLs and most of the SM in the Golgi apparatus, Cer must travel from one compartment to the other. Due to its hydrophobic nature, Cer do not spontaneous transfer through the cytosol, but need facilitated mechanisms. The transport of Cer to the Golgi occurs either through the action of a lipid transfer protein (CERT), which specifically delivers Cer for SM synthesis, or through a vesicle‐mediated route. Vesicular transport delivers Cer mostly for the synthesis of GluCer, but also SM biosynthesis is partially dependent on the vesicular transport of a pool of Cer to SM synthase (Riboni et al., 2010). Cer can also undergo phosphorylation of the hydroxyl group of C-1 by the Cer kinase (CK), forming Cer-1-phosphate (Cer1P) (Hannun and Obeid, 2008). The subcellular localization of this enzyme has not been fully defined yet, but it seems to be at the level of the plasma membrane, the Golgi apparatus, and the cytoplasm (Van Overloop et al., 2006).

Intermediate and final products of sphingolipid biosynthesis reach the plasma membrane mostly via the vesicles following the exocytotic membrane flow (Riboni et al., 2010).

An important point to be taken into account concerns the dynamic state of the plasma membrane. Non dividing cells are characterized by a high degree of membrane internalisation and recycling. They are estimated to internalize via endocytosis about half their plasma membrane per hour. In this process, membrane components (lipids and proteins) are, at least in part, degraded and resynthesized or recycling, in order to maintain the cell surface composition dynamically constant.

Consequently, the turnover of SLs has to be rapid in order to correspond to the overall membrane turnover, as well as to assist the formation of sphingoid bioregulators (Tettamanti et al., 2003).

Membrane GSLs are constitutively degraded by a process involving endocytosis and the endo-lysosomal district. The intralysosomal degradation proceeds by the action of soluble hydrolytic enzymes, the (exo)glycohydrolases, which sequentially cleave off individual sugar residues starting from the non-reducing end of their glycolipid substrates. These enzymes are assisted by effector protein molecules named "sphingolipid activator proteins (SAPs, or saposines)" and negatively charged lipids. All the enzymatic steps of the degradative process require an acidic pH inside the organelle, that is warranted into the late endosome/lysosome organelles by the action of a proton pump that brings $H^+$ from the cytosol to the inside of the organelles. The sequence of sugar removal from gangliosides is as follows: (a) the lysosomal sialidase convert the multi-sialogangliosides into the corresponding mono-sialogangliosides GM1and GM2 (which are resistant to this enzyme), or LacCer (from GM3); (b) galactose is removed from GM1 by a β-galactosidase (in the presence of either the GM2-AP or SAP-B) to form GM2; from GM2 the N-acetyl-galactosamine residue is split off to produce GM3 by the action of β-N-acetyl-hexosaminidase, which requires GM2-AP, an activator that is essential for the in vivo degradation of the GM2 gangliosides; (c) in some cells and animals, specific sialidases (GM1- and GM2-sialidase) remove sialic acid from GM1 and GM2, producing the corresponding a-sialo derivatives GA1 and GA2. GA1 and GA2 are then converted to LacCer by the action of β-galactosidase and β-N-acetyl-hexosaminidase or only β-N-acetyl-hexonaminidase; (d) finally, a β-galactosidase (in the presence of either SAP-B or -C) and a β-glucosidase act in sequence to degraded LacCer into Cer. The final products of degradation (individual monosaccharides, long chain bases, fatty acids), as well as intermediate by-products (for instance, LacCer, GlcCer and Cer) can leave the late endosome/lysosome and enter in the cytosol, where they can be "re-used" in biosynthetic pathway of SLs ("salvage pathway") or can be further degraded (Tettamanti et al., 2003). As regard Cer and SM, both lysosomal and non-lysosomal degradation steps are known, which apparently do not need the assistance of an activator protein. SM is catabolized by the action of the enzyme sphingomyelinase (SMase), which hydrolyze the SM phosphodiester bond, with the consequent formation of Cer and phosphocholine. Three isoforms of SMase (acidic, neutral, and alkaline) have been identified and distinguished, according to their subcellular localization and the optimum pH of activity. Different enzymatic isoforms of acidic SMase exist and are localized in different cellular and extracellular compartments. Indeed, acidic SMase can reside in the lysosomal compartment, be secreted, or be in close association with the outer leaflet of the PM, where it can form Cer-rich platforms (Schissel et al., 1998; Stancevic and Kolesnick, 2010; Tabas, 1999). The neutral SMase (N-SMase) has distinct subcellular locations, including the inner leaflet of the plasma membrane, the ER, the Golgi, and even the nucleus (Clarke et al., 2006; Marchesini and Hannun, 2004). The alkaline SMase is mostly expressed in the

intestinal tract and in the bile, and is involved in SM digestion (Duan, 2006). Cer are also produced by dephosphorylation of Cer1P by lipid phosphatase(s). Cer can be catabolized by the action of specific a ceramidases (CDases), which hydrolytically cleave the N-acyl linkage of Cer to form sphingosine (Sph) and free fatty acid. Three isoforms of CDases, with different optimum pH and subcellular localizations, have been identified: acidic, neutral and alkaline CDases, which are located at the plasma membrane, lysosome, and ER/Golgi complex, respectively (Mao and Obeid, 2008). Cer-derived Sph can be recycled or undergo phosphorylation in position C1 with the production of S1P by the action of Sph kinases (SKs). Two mammalian isozymes, which are known as SK1 and SK2, have been characterized. SK1 is predominantly cytosolic (Wattenberg et al., 2006) and SK2 is cytosolic and also associated with the nucleus (Igarashi et al., 2003). S1P can be irreversibly catabolized into hexadecenal and phosphoethanolamine by the action of S1P lyase (located on the ER-cytosolic side) in the final step of SL catabolism (Serra and Saba, 2010). Alternately, S1P can also be dephosphorylated back to Sph by the action of either S1P specific phosphatases, or non-specific lipid phosphate phosphatases (Le Stunff et al., 2007; Le Stunff et al., 2002; Pyne et al., 2009). Notably, Sph origin is exclusively catabolic, since it only derives from SLs degradation (Sandhoff and Kolter, 1996). Sph can be recycled for SL formation via a recycling or salvage pathway (Hannun and Obeid, 2008). Indeed, Sph can reach the ER where it is N-acylated to regenerate Cer that can be used for the biosynthesis of complex SLs. The Sph recycling process provides an energy advantage to cells. It has been shown that in different cell types, such as cells of glial origin, it may constitute the principal pathway of SLs synthesis (Tettamanti et al., 2003).

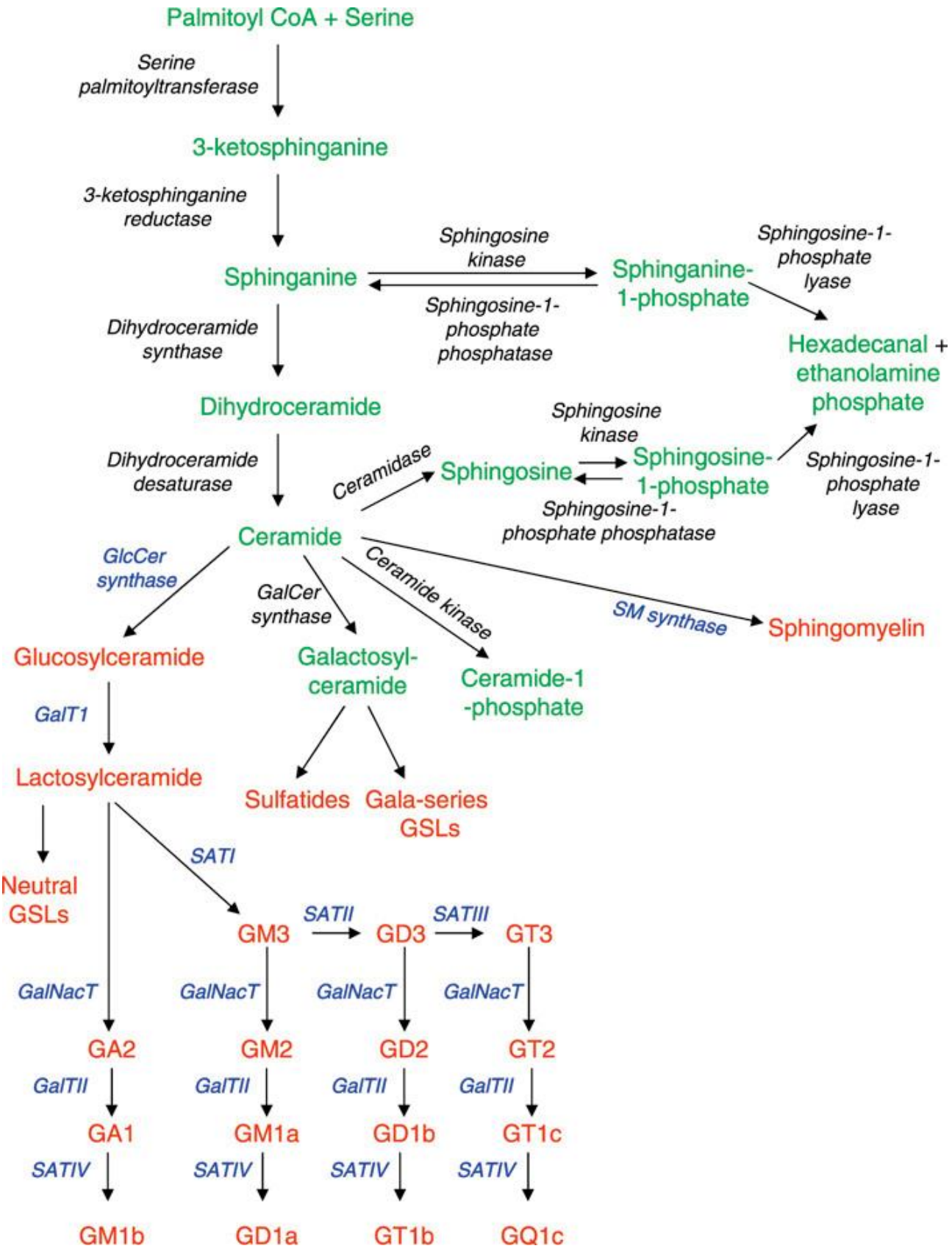


**Figure 2.** *Schematic representation of SLs metabolism.* SLs synthesized in the ER are in green, and those synthesized in the Golgi apparatus are in red. Enzymes are shown in italics, with those that reside in the ER in black and those that reside in the Golgi apparatus in blue. GalNacT, N-acetylgalactosamine transferase; GalT, galactosyltransferase; SAT, sialyl transferase (Lahiri and Futerman, 2007).

## 3. ANTITUMOR ALKYLPHOPSHOLIPIDS

### 3.1 An overview of antitumor Alkylphospholipids

Alkylphopsholipids (APLs) are lipid analogues that were first synthesized as potential immune modulators in the late 1960s, based on lysophosphatidylcholine (lysoPC) structure (Eibl et al., 1967). Soon after, their role as antitumor agents was discovered (Eibl and Unger, 1990). At the beginning of their synthesis, the ester linkages at C1 and C2 of the glycerol backbone of lysoPC were replaced by ether bonds in order to yield metabolically stable analogs (van Blitterswijk and Verheij, 2013). Among this first generation of APLs, there is Edelfosine, which contain also a methyl group linked through an ether bond at the C2 position. Many other APLs were subsequently synthesize as promising anticancer agents, modifying Edelfosine chemical structure with the aim to improve metabolic stability and therapeutic potency. Although conventionally these compounds are collectively called APLs, formally they are divided in two classes: alkyl-lysophospholipids, which maintain the glycerol backbone, and alkyl-phospholipids, which are characterized by the lack of the glycerol backbone and by the alkyl chain esterified directly to the phosphobase (Fig. 3). Edelfosine and its thio-ether derivative Ilmofosine belong to the first class, while Miltefosine, Perifosine (PF), Erucylphosphocholine, and Erufosine belong to the second class (van Blitterswijk and Verheij, 2013). Edelfosine and Ilmofosine, both first generation of APLs, show a relatively low stability compared to the APLs of the second class, due to the glycerol backbone (Fensterle et al., 2014). Although Edelfosine has shown selective cytotoxicity toward tumor cells both in vitro (Mollinedo et al., 2010; Mollinedo et al., 1997; Ruiter et al., 1999; Runge et al., 1980) and in preclinical models (Berdel et al., 1980; Berger et al., 1984; Mollinedo et al., 1997; Scherf et al., 1987; Tarnowski et al., 1978), it has proven disappointing in clinical trials. In fact, the clinical use of Edelfosine at the moment is substantially restricted to the bone marrow purging ex vivo in acute leukemia patients (Vogler et al., 1996; Vogler et al., 1992). Similarly, Ilmofosine has displayed anti-cancer activity both in vitro and in vivo against different type of cancers, but results from clinical trials have been discouraging (Giantonio et al., 2004; Herrmann et al., 1990; Woolley et al., 1996). Miltefosine, the minimal structural requirement for the anti-tumor activity of APLs, is a second generation APL that has shown potent antitumor activity in vitro such as in some tumor models, and is currently used for the topical treatment of cutaneous breast cancer metastases (Leonard et al., 2001) and cutaneous lymphomas (Dumontet et al., 2006). Because of its hemolytic effect when injected intravenously, it can only be administered orally or topically (Kotting et al., 1992). Unluckily, dose limiting toxicities impeded its further development as an oral anti-cancer agent. The third generation APLs, Perifosine, Erucylphosphocholine, and Erufosine, were synthesized as variants of Miltefosine. In

Perifosine, the choline headgroup of Miltefosine is replaced by a heterocyclic methylated piperidine moiety, giving rise to a markedly stable molecule, with fewer gastrointestinal side effects (Hilgard et al., 1997; Vink et al., 2005). Perifosine has turned out to be an orally-active agent with potent antineoplastic activity and is currently tested in clinical trials for the treatment of a wide variety of cancer types, as we will talk about extensively later on (Fensterle et al., 2014). Erucylphosphocholine consists of a carbon chain longer than Miltefosine and presents a *cis*-13,14 double bond. Erufosine differs from Erucylphosphocholine only by the introduction of a methyl residue in the choline headgroup, making this agent more soluble in aqueous solutions. These structural modifications eliminate hemolytic- and myelotoxicity due to the formation of lamellar instead of micellar structures in aqueous solutions, allowing these compounds to be administrated intravenously, reducing gastrointestinal toxicity and reaching a higher plasma concentrations in vivo (van Blitterswijk and Verheij, 2013). Preclinical studies, both in vitro and in vivo, have shown that these two synthetic lipids are promising therapeutic agents against brain tumor. Indeed, they are effective even at relatively low doses and, compared to the others APLs, have a greater ability to efficiently cross the blood brain barrier and to accumulate in brain tissue (Erdlenbruch et al., 1999; Erdlenbruch et al., 1998; Henke et al., 2009). At the moment, there aren't clinical data available on these compounds.

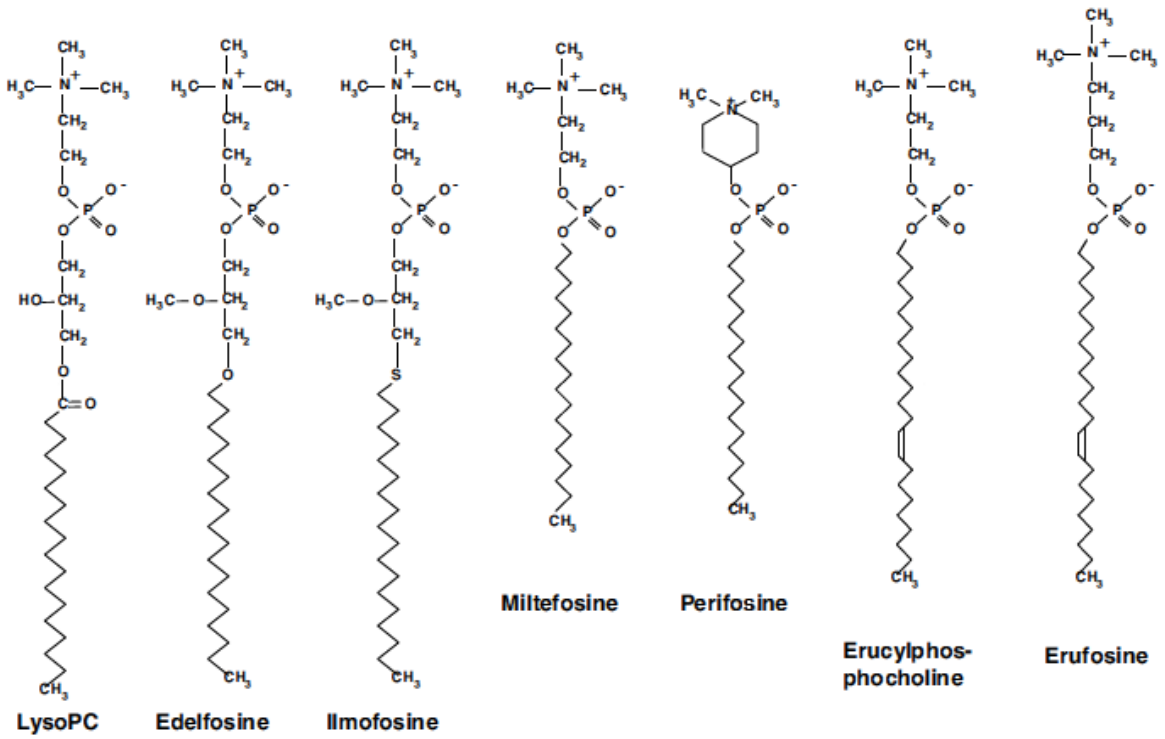


**Figure 3.** *Chemical structures of LysoPC, Edelfosine, Ilmofosine, Miltefosine, Perifosine, Erucylohosphocholine, and Erufosine* (modified image from van Blitterswijk and Verheij, 2013).

Recently, a novel class of APLs that displays significant anticancer activity has been synthesized with the primary aim to improve solubility in aqueous solutions. This is the class of glycosylated antitumor ether lipids (GAELs), the fourth generation of APLs, that are in turn divided into two main subclasses: glycosidated phospholipids and non-phosphorous GAELs. Glycosylated phospholipids contain carbohydrates or carbohydrate-related molecules at the chemical lead of a biologically active phospholipid, the platelet-activating factor (PAF), whose chemical structure exhibits similarity to Edelfosine and was identified ten years after Edelfosine synthesis (Fig. 4). Inositol-C2-platelet-activating factor is the most potent anticancer agent of this class. Non-phosphorous GAELs have a carbohydrate moiety in place of the phosphobase present in APLs. Among this group, there is ET-16-$OCH_3$-Gln, the most effective compound of this new family to date. The antitumor activities showed both in vitro and in vivo make these compounds promising treatments to test in clinical trials (Arthur and Bittman, 2014; Semini et al., 2014).

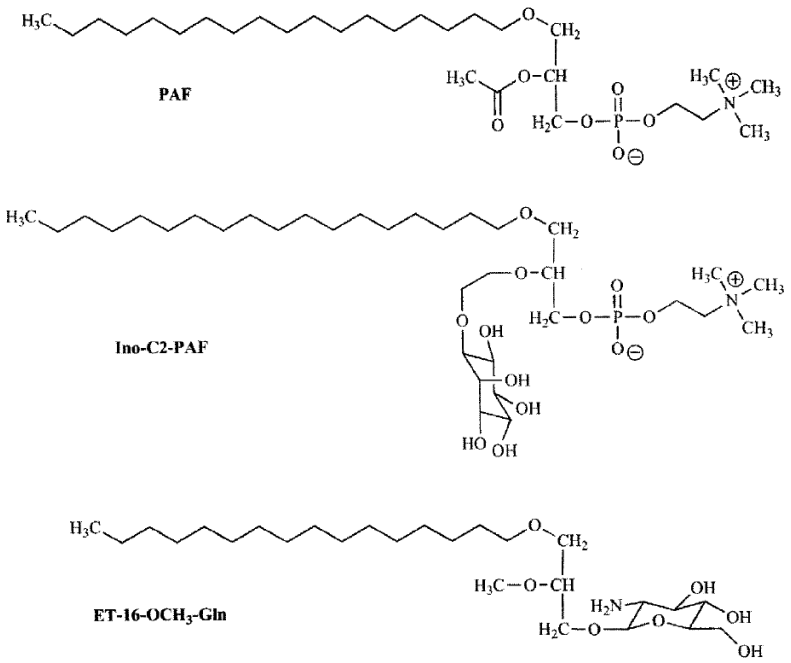


**Figure 4.** *Chemical structures of PAF, Ino-C2-PAF, and ET-16-$OCH_3$-Gln* (modified image from Mollinedo, 2014).

## 3.2 Alkylphospholipids in cancer: mechanisms of action, modes of internalization and cellular resistance

The mechanisms of action of APLs are different from that of standard chemotherapeutic drugs. Indeed, APLs do not interact directly with the DNA, but rather target the cell membranes where they accumulate, due to their aliphatic character and metabolic stability. At high concentrations, they would cause cell lysis, owing to their detergent properties. Nevertheless, at lower clinically achieved concentrations, they have an impact on membrane lipid composition and lipid metabolic

processes, affecting lipid-based signal transduction pathways which can lead to cellular stress, cell growth inhibition and/or apoptosis. APLs show higher specificity towards metabolically active/proliferating cells, like tumor cells, than towards quiescent normal cells (van Blitterswijk and Verheij, 2013). A wide variety of mechanisms have been proposed to clarify the actions of these compounds. However, it is still unclear what molecular target play a crucial role for their ability to induce cell growth arrest or cell death and may well differ according to cell type and the type of ALP (van Blitterswijk and Verheij, 2013). Extensive evidence suggests that the inhibition of phosphatidylcholine (PC) biosynthesis by APLs is a key event determining inhibition of cell proliferation and/or apoptosis in different tumor cell lines. Indeed, PC is the most abundant phospholipid in eukaryotic cellular membranes, not only essential for new membrane formation, but also involved in cell signaling processes. APLs inhibit the rate-limiting enzyme for PC biosynthesis, CTP:phosphocholine cytidylyltransferase (CT). This enzyme resides in the nucleus and cytoplasmic compartments, where it is inactive, and becomes active when it is bound to membranes. APLs prevent CT translocation from the cytosol to the membrane of the ER. In particular, it has been suggested that APLs, that are type I lipids, impair the stored curvature elastic energy of the membrane, leading to membrane expansion with the consequent reduction in the partitioning of CT in the membrane (Dymond et al., 2008). How the inhibition of PC synthesis initiates apoptosis is still unclear. One possibility is that the shortage of PC inhibits the SM biosynthesis catalyzed by SMS from PC and Cer, leading to an accumulation of Cer, which is a pro-death molecule (Morad and Cabot, 2013). Another possibility is that the inhibition of PC synthesis in the ER triggers ER stress and/or oxidative stress, e.g. inducing the ER stress-related pro-apoptotic transcription factor CHOP/GADD153, which subsequently lead to the activation of the apoptotic machinery (van Blitterswijk and Verheij, 2013). Other authors, however, consider that the inhibition of PC synthesis is not a crucial event, but only plays a minor role in APLs-induced cell death (van der Sanden et al., 2004). Different studies have demonstrated that in several tumor cell lines APLs are enriched in membrane microdomains (also known as lipid raft) and that their antineoplastic activity is closely related with these structures. It has been proposed that their mode of action might be related with their ability to induce alterations in lipid composition and/or biophysical properties of lipid rafts (Castro et al., 2013; Marco et al., 2014; van Blitterswijk and Verheij, 2013). Lipid rafts are specific membrane microdomains enriched in cholesterol and saturated SLs that are more ordered than the bulk membrane (Lingwood and Simons, 2010). Growing evidence suggests that lipid rafts, with their unique lipid composition, provide specialized lipid environments, which regulate the sorting of membrane proteins, and the recruitment and the concentration of signaling molecules at the plasma membrane level, functioning in membrane signaling and trafficking (Coskun and Simons, 2010;

Simons and Gerl, 2010). Within this context, it has been established that cholesterol is crucial in stabilizing different types of lipid microdomains (Bakht et al., 2007; Pike, 2009) and, coherently, it has also been shown that enrichment of the membrane with cholesterol destabilizes membrane rafts (Marco et al., 2014). Interestingly, APLs could induce a deregulation of cholesterol homeostasis. In particular, APLs impair the non-vesicular transport of cholesterol from the plasma membrane to the ER, inhibiting cholesterol esterification. This causes a significant cholesterogenic response and so an increase in the *de novo* synthesis and uptake of cholesterol, that ultimately leads to an accumulation of free cholesterol within the cell and, in particular, in membrane microdomains such as lipid rafts. So, it has been suggested that the alteration of cholesterol homeostasis induced by APLs, concomitant to the reduction in PC and SM biosyntheses, leads to a modification in the cholesterol/choline-bearing phospholipids ratio, affecting lipid rafts composition and so their integrity and functionality. The APLs-induced disturbance of the cell native membrane structure may lead to alterations of signaling pathways critical for cell survival and growth (Gajate and Mollinedo, 2014; Marco et al., 2014). Mollinedo and coworkers have shown that in several hematologic cancer cells APLs (Edelfosine in particular) induce apoptosis by the coaggregation of Fas/CD95 death receptor with lipid rafts, independent of its natural ligand FasL, driving the formation of the so called DISC complex (death-inducing signaling complex), together with the recruitment into lipid rafts of downstream signaling molecules. The authors coined the term CASMER (cluster of apoptotic signaling molecule-enriched rafts) to indicate this novel raft-based supramolecular entity, which acts as death-promoting scaffolds facilitating the triggering of a potent cell death response (Gajate and Mollinedo, 2014). The action of APLs on lipid rafts does not only favor cell death pathways, but also alters survival, adhesion and migration signal transduction pathways (Mollinedo, 2014). It has also been shown that APLs may accumulate in mitochondria, causing their swelling and destabilization. Mitochondria are crucial components of cell's apoptosis machinery. So, APLs may initiate apoptosis altering mitochondrial membranes (Kuerschner et al., 2012; Mollinedo et al., 2011).

To affect their multiple cellular targets, the APLs have to be internalized into the cells. Essentially two modes of internalization are possible: (a) by lipid rafts-mediated endocytosis and (b) by trans-bilayer movement from the outer to the inner leaflet of the plasma membrane. The last occurs either via spontaneous flipping (slow because energetically unfavorable), or through an ATP-dependent lipid translocator. It has long been difficult to determine which of the two mechanisms is most relevant and it seems to depend on the cell types (van Blitterswijk and Verheij, 2013). Indeed, lymphoid cells internalize ALPs predominantly via lipid rafts-mediated endocytosis, while carcinoma cells utilize a lipid translocator/flippase (Munoz-Martinez et al., 2008; Vink et al., 2007).

Alteration of the mechanisms of APLs internalization can cause APL-mediated apoptosis resistance. In carcinoma cells, e.g., APLs uptake occurs through a transmembrane phopsholipids flippase P4-ATPase ATP8B1, which function in complex with a CDC50a subunit. The CDC50a subunit is not only necessary for ATP8B1 functionality, but also for its export from the ER to the plasma membrane. Accordingly, it has been shown that in PF-resistant carcinoma cells a defect in CDC50a trafficking and plasma membrane insertion is the underlying mechanism of PF-resistance (Munoz-Martinez et al., 2010). On the other hand, S49 mouse lymphoma cells made resistant to ALP (Edelfosine), $S49^{AR}$ cells, showed a complete downregulation of SMS1 and consequently were not able to synthesize SM (Van der Luit et al., 2007a), a major component of lipid rafts, where APLs have been shown to accumulate (van Blitterswijk and Verheij, 2013). These cells had acquired cross-resistance to other ALPs (van der Luit et al., 2002; Van der Luit et al., 2007a; van der Luit et al., 2007b; Vink et al., 2007) as well as to DNA damage and Fas/CD95 ligation (Alderliesten et al., 2011; van Blitterswijk et al., 2010). Accordingly, $S49^{AR}$ cells exhibited a decreased Fas/CD95 expression (van Blitterswijk et al., 2010), a sustained ERK/Akt activity (Alderliesten et al., 2011), and an abrogated raft-mediated ALP internalization (van der Luit et al., 2002). SM synthesis by SMS1 in the Golgi is essential for new lipid raft formation and for the anterograde (from the Golgi towards the plasma membrane) and the retrograde (from the plasma membrane to the Golgi) vesicular trafficking (Ikonen, 2001; Tafesse et al., 2006; van Blitterswijk et al., 2003). SMS1 deficiency would prevent ALP internalization, blocking raft endocytosis along the retrograde route, and so make cells resistant. Interestingly, in parental S49 cells downregulation of SMS1 by siRNA was enough to induce the above-mentioned cross-resistance (Van der Luit et al., 2007a; van der Luit et al., 2007b). It was also found that $S49^{AR}$ cells lacked the $PI(3,4,5)P_3$ phosphatase SHIP-1 [SH2 (Src homology 2)-domain-containing inositol phosphatase 1], a known regulator of the Akt survival pathway (Alderliesten et al., 2011). It was suggested that SHIP-1 cooperate with SMS to regulate apoptosis sensitivity of lymphoma cells to ALPs, as well as to other apoptotic stimuli. Indeed, re-sensitization of the $S49^{AR}$ cells, by prolonged culturing in the absence of ALP, restored SHIP-1 expression as well as phosphoinositide and SM levels. Knockdown of SHIP-1 in parental cells mimicked the $S49^{AR}$ phenotype in terms of apoptosis cross-resistance, SM deficiency and altered phosphoinositide levels (Alderliesten et al., 2011). So, the sensitivity to ALPs could be very different among several cancer cell types and depend on the different contributions of pro- and anti-apoptotic mediators, as well as on the rate of cellular ALPs uptake, which, in turn, has been shown to be closely related to the extent of cellular proliferative activity and associated metabolic (lipid) turnover (van Blitterswijk and Verheij, 2013). As regards their clinical use, APLs are among the most promising candidates to combine with other anticancer therapy. These compounds have the

advantage of possessing a unique mechanism of action, acting at the cell membranes levels rather than on the DNA, as many conventional therapies. So, it is conceivable that a combination of drugs with different mechanism of action may lead to additive or even synergistic therapeutic effects and may also overcome chemo- and radio-resistance of tumor cells. The most studied and most successful ALP in combination therapies is PF (van Blitterswijk and Verheij, 2013).

### 3.3 Perifosine

PF is a third generation APL, composed of a heterocyclic piperidine group connected by a phosphate group to a long alkyl chain. PF exhibited a potent antineoplastic activity against a wide range of tumor cell types in vitro and a high specificity against cancer cells, as it has been shown in pre-clinical and clinical studies (Fensterle et al., 2014; van Blitterswijk and Verheij, 2013; Vink et al., 2005). The PF-enrichment in tumor tissues might be mediated by a selective enrichment in cholesterol rich lipid rafts of tumor cells (Fensterle et al., 2014). PF is both a cytostatic and a cytotoxic agent (Fensterle et al., 2014). Compared to the other previously synthesized APLs, this novel oral APL showed increased stability and, consequently, a prolonged plasma half-life in vivo (Hilgard et al., 1997; Vink et al., 2005). The long plasma half-life and the substantial accumulation of PF in tumor tissues allowed clinical dosing regimens with moderate oral doses, resulting in limited toxicity. The favorable toxicity profile of PF is a fundamental prerequisite for its use in combination with other anticancer drugs (Gills and Dennis, 2009). PF was tested in a multitude of clinical studies both as single agent and in combination with other agents (Gills and Dennis, 2009; Vink et al., 2005). Clinical trials of PF as a single agent on recurrent prostate cancer, adenocarcinomas, and melanomas have been disappointing (Vink et al., 2005), while on renal cell carcinoma and neuroblastoma they have shown promise (Porta and Figlin, 2009 ; Belcher et al., 2010). However, PF anticancer activity has been most successfully exploited in combination with other anti-cancer regimens. Combination trials in multiple myeloma and colorectal cancer have reached Phase III, but recently these studies failed to demonstrate clinical activity with the low dose PF scheme in patients with metastatic colorectal cancer and relapsed multiple myeloma in combination with capecitabine and bortezomib, respectively (Fensterle et al., 2014). PF has different targets which have been linked to its anticancer activity (Fig. 5). To date, the Ser/Thr kinase Akt, which is a key regulator of multiple survival pathways, is considered as the most important molecular target of PF (Fensterle et al., 2014; van Blitterswijk and Verheij, 2013). Akt is a central member of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which is aberrantly active in most human malignancies and positively regulates cell growth, proliferation,

and survival. Furthermore, PI3K/Akt pathway activation has been associated to therapeutic resistance. So, the Akt pathway is assumed to be one of the most attractive targets for tumor therapy (Fu et al., 2009; LoPiccolo et al., 2008). The mechanism by which PF inhibits Akt is very different from that of previous ATP-competitive and allosteric Akt-inhibitors. PF interferes with the normal function of the lipid-binding pleckstrin homology (PH) domain of Akt. PF may do so in two different ways: (1) by disrupting membrane microdomains crucial to Akt activation and (2) by binding directly to the PH domain of Akt, thereby displacing the natural $PI(3,4)P_2$ and $PI(3,4,5)P_3$ ligands. Since PF prevents the Akt recruitment to the plasma membrane, Akt is no longer able to adopt the favorable conformation for its phosphorylation/activation and to activate its downstream effectors (Bellacosa et al., 1998; Dineva et al., 2012; Gills and Dennis, 2009; Hennessy et al., 2007; Kondapaka et al., 2003; van Blitterswijk and Verheij, 2013). Coherently, overexpression of myristoylated Akt that is constitutively active (it does not require $PI(3,4)P_2$ and $PI(3,4,5)P_3$ bindings for the plasma membrane recruitment) abolished PF-mediated decrease of Akt phosphorylation and cell growth inhibition/apoptosis (Gills and Dennis, 2009; Hennessy et al., 2007; Kondapaka et al., 2003; van Blitterswijk and Verheij, 2013). PF-mediated Akt inhibition may also modulate the activity of many important downstream targets. As an example, PF induces cell cycle arrest through the up-regulation of $P21^{WAF1/CIP1}$ in a p53 independent manner and the loss of cyclin-dependent kinase 2 (cdk2) activity (Kondapaka et al., 2003; Patel et al., 2002). Furthermore, Fu et al. showed that PF in human lung cancer cell lines, besides inhibiting Akt, inhibited mTOR signaling (Fu et al., 2009). In particular, PF hindered the assembly of both mTOR/raptor and mTOR/rictor complexes by promoting the degradation of major elements in the mTOR axis, including mTOR, raptor and rictor. As a consequence, PF induced autophagy in addition to apoptosis. The combination of PF with lysosomal inhibitors enhances apoptosis induced by PF as well as its anticancer efficacy in vivo, suggesting that PF-induced autophagy protects cells from undergoing apoptosis (Fu et al., 2009; Sun, 2010). It was also shown that PF, through Akt-inhibition, prevent the inhibitory phosphorylation of GSK3β which, in turn, modulates the activity of the Wnt signaling pathway (Floryk and Thompson, 2008; Korkaya et al., 2009). Finally, in waldenstroem macroglobulaemia cells, PF inhibited Akt and NF-kB, which can be activated by Akt via the Akt-IKK-IkB- NF-kB axis. Akt is a validated target of PF at pharmacological relevant levels, since Akt inhibition by PF has been confirmed in a wide variety of tumor cell lines as well as in vivo in xenograft models of different type of cancer (Fensterle et al., 2014). In particular, the most persuasive preclinical study relating PF-induced Akt inhibition with anticancer potency was performed by Hennessy et al. In this study, the authors confirmed that in different tumor cells PF inhibit Akt activation by hindering its translocation to the plasma cell membrane and demonstrated that in several prostate, breast and

ovarian cancer xenograft models, growth inhibition was correlated with the extend of Akt inhibition induced by PF administered on different schedules. They also demonstrated that cell lines resistant to PF did not show Akt inhibition in vivo (Hennessy et al., 2007). However, PF exerts also Akt-independent effects and the importance of Akt inhibition for its anticancer activity would still be an open question (Gills and Dennis, 2009). Indeed, although many preclinical studies have reported Akt inhibition by PF, only few clinical trials with PF have evaluated Akt status in patient tissues, rendering difficult to make assumptions on the contribution of Akt to its clinical efficacy. As discussed extensively as regards the mechanisms of action of APLs in general, PF, in addition to targeting Akt, interferes with the biosynthesis of membrane lipids, e.g. inhibiting PC biosynthesis (van Blitterswijk and Verheij, 2008), and altering the homeostasis and transport of cholesterol (Carrasco et al., 2010; Jimenez-Lopez et al., 2010; Rios-Marco et al., 2013). Furthermore, PF might disturb membranes both acting on the metabolism and transport of membrane lipids and/or directly inducing a biophysical disturbance of cell membranes in which it is inserted, consequently affecting membrane lipid turnover and membrane associated signaling events, including Akt signaling (van Blitterswijk and Verheij, 2013). Recent studies have also reported that PF increased endogenous Cer level to mediate cell apoptosis. In particular, it has been shown that PF enhanced Cer production when administered in combination with histone deacetylase inhibitors (HDACi) (Rahmani et al., 2005), curcumin (Chen et al., 2012), paclitaxel (Sun et al., 2011), and UVB radiation (Ji et al., 2012). However, in some cell lines, PF-induced Cer production is subject to metabolic clearance, and is not enough to promote cell death (Yao et al., 2013). In a human glioblastoma cell line (U87MG), it has been shown that PF only slightly enhanced Cer production, while exogenously added short-chain Cer (C6) inhibited PF-induced protective autophagy to restore cell apoptosis and PF sensitivity (Qin et al., 2013). Interestingly, in non-small cell lung cancer cell lines PF, through SMase-mediated generation of Cer, stimulated the release of nanovesicles containing molecules involved in cell signaling, including active Akt, EGFR (epidermal growth factor receptor), and IGF-IR (insulin-like growth factor receptor), decreasing their cellular levels. The increase of Cer determined by PF also induced cell detachment and death. Furthermore, when transferred in vitro, PF-induced nanovesicles increased Cer levels and death in recipient cells. These findings indicate that in these cells Cer generation underlies the Akt inhibition and cytotoxicity of PF, and suggest nanovesicles shedding and uptake might potentially propagate their cytotoxicity in vivo (Gills and Dennis, 2009). There are also many PF targets in signal transduction pathways, in addition to that mentioned above, which might contribute to its mechanism of action. These targets may be either primary or secondary targets, probably depending on cellular context (Fensterle et al., 2014). One of these targets is the Jun N-terminal kinase (JNK). Several pathways, including cellular

stress, can activate JNK, which exerts a pro-apoptotic activity e.g. by phosphorylating and thus inactivating the antiapoptotic protein Bcl-$X_L$. The activation of JNK has been shown to be important for PF-induced apoptosis (Carrasco et al., 2010; Chiarini et al., 2008; Gajate et al., 1998; Li et al., 2006; Papa et al., 2008; Ruiter et al., 1999). PF was also shown to reduce the expression of survivin and xiap, which are essential pro-survival proteins of the iap family, while increasing at the same time the expression of the proapoptotic trail receptor/death-receptor 5 (Fensterle et al., 2014). Finally, some authors have shown that PF can have either an inhibitory effect on Erk, a member of the Ras/Raf/Mek/Erk cascade (Fei et al., 2010; Papa et al., 2008; Pinton et al., 2012), or leads to its activation (Hideshima et al., 2006; Hou et al., 2014; Leleu et al., 2007). These opposing results may indicate that modulation of Erk activity plays only a minor role in the anticancer activity of PF as compared to other PF-effects constantly observed for a large number of different cell types (Fensterle et al., 2014).

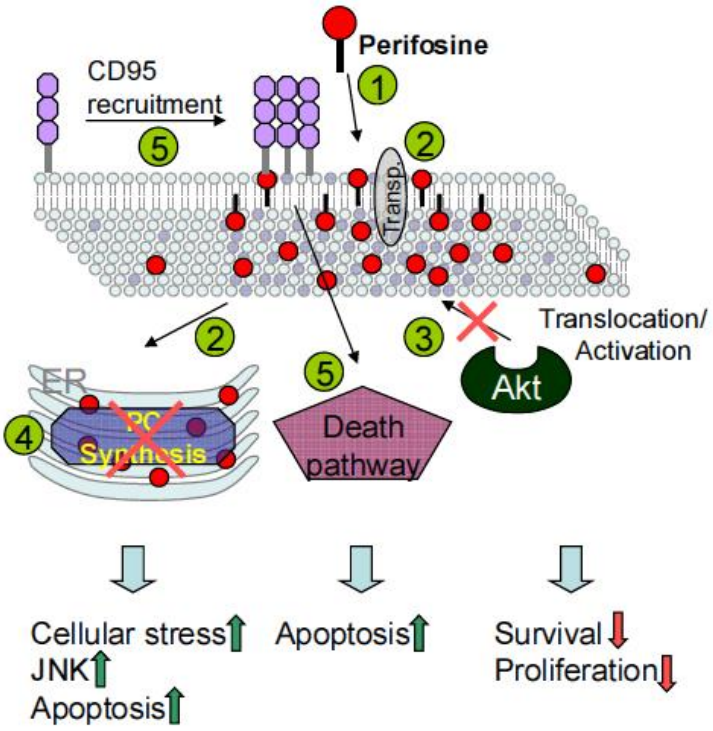


**Figure 5.** *Current model of central components of the mechanism of action of PF* (Fensterle et al., 2014).

# AIM OF THE WORK

GBM is the most frequent malignant primary brain tumor in adult and one of the most lethal human cancers, presenting one of the greatest challenges in oncology. Despite the use of the latest surgical, radiation, and chemotherapy treatments, patients with GBM have a poor prognosis, with a median survival rate of only 15 months (Rich and Bigner, 2004; Wen and Kesari, 2008). Indeed, GBM anatomical location and its complex and heterogeneous biology enormously complicate the use of conventional therapies and the development of new treatments (Holland, 2000; Mrugala, 2013). PF is a synthetic lipid analogue belonging to a relatively new class of structurally related antitumor agents: the APLs. These compounds constitute appealing candidates to develop novel therapeutic approaches to treat cancer. Indeed, the mechanisms of action of APLs are different from that of standard chemotherapeutic drugs. APLs do not interact directly with the DNA, but rather target the cell membranes, where they preferentially accumulate due to their amphiphilic nature (van Blitterswijk and Verheij, 2013). Mounting evidence demonstrates that PF exerts a marked cytotoxic effect on a wide range of human cancers, both *in vitro* and *in vivo*, and is currently being tested in phase II clinical trial against major human tumors (Fensterle et al., 2014; Gills and Dennis, 2009). However, the effect of PF against gliomas is poorly investigated. Accumulating literature suggests that PF could act on tumor cells eliciting apoptosis and/or cell growth arrest. More in detail, it has been indicated that the inhibition of the survival signaling pathway PI3K/Akt, which is frequently up-regulated in GBM cells, is one of the main mechanism by which PF exerts its antitumor effects (van Blitterswijk and Verheij, 2013). However, besides Akt, PF affects other several important cellular targets and so the relevance of Akt inhibition for its activity would still be an open question (Fensterle et al., 2014; Gills and Dennis, 2009). As other ALPs, PF may disturb cell membranes, provoking a broad range of biological effects, ultimately leading to cell death. Indeed, PF may induce a biophysical disturbance of cell membrane in which it is inserted, affecting membrane lipid turnover and membrane associated signaling events (including Akt signaling) and/or interfere with the metabolism and transport of membrane lipids, thus affecting cell membranes composition and functionality (Marco et al., 2014). In particular, it has been shown that PF could modify the properties of lipid rafts (Fensterle et al., 2014), ordered membrane lipid domains, enriched in cholesterol and SLs, serving as sorting platforms and hubs for signal transduction proteins (Sonnino and Prinetti, 2013). Numerous signaling pathways involved in cancer development and progression, such as cell adhesion, migration, as well as cell survival and growth, are dependent on these structures (Mollinedo and Gajate, 2015). It has been well established that SLs exert a key role in the formation, stabilization, and function of lipid domains (Sonnino and Prinetti, 2013). Therefore,

altered SLs content and/or altered ratio between SLs and glycerophospholipids could influence lipid membrane domains formation and behavior.

On these premises, the aim of this PhD project was to investigate the effect of PF on cell survival in different GBM cells, and to provide a contribution to the understanding of its molecular mechanism, evaluating the ability of PF to target membrane lipid metabolism and content, that ultimately can affect membrane-related signaling pathways crucial in the regulation of cell demise.

# MATERIALS AND METHODS

## MATERIALS

All reagents were of the highest purity available. DMEM, L-glutamine, streptomycin, penicillin, amphotericin-B, sodium pyruvate, aprotinin, leupeptin, pepstatin, bestatin, fatty acid free bovine serum albumin (BSA), O-Tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt (D609), 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), 2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride (Hoechst 33342), 3,6-bis[dimethylamino]acridine (acridine orange), primary rabbit anti-LC3B antibody, Kodak Biomax film and other common chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was from EuroClone (Pero, Milan, Italy). D-erythro-[$^{3}$H]-sphingosine ([C3-$^{3}$H]Sphingosine, 21.4 Ci/mmol), [methyl-$^{3}$H]Choline Chloride ([methyl-$^{3}$H]Choline, 75 Ci/mmol), Ultima Gold were from PerkinElmer Life Sciences (Boston, MA, USA). LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1benzopyran-4-one) was from Vinci-Biochem (Firenze, Italy). The Primary antibody recognizing phospho-Akt (Ser-473) were from Cell Signaling Technology, Inc. (Danvers, MA). Goat anti-mouse horseradish peroxidase-linked secondary antibody was from Thermo Fisher Scientific (Waltham, MA, USA). Primary rabbit anti-GAPDH antibody, primary rabbit anti-pERK1/2 antibody, primary mouse anti-ERK2 antibody, and Goat anti-rabbit horseradish peroxidase-linked secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). High performance thin layer chromatography (HPTLC) silica gel plates and all solvents were from Merck (Darmstadt, Germany). SuperSignal West Pico and West Femto Maximum Sensitivity Chemioluminescent Substrate and bovine serum albumin (BSA) fraction V were purchased from Pierce Chemical Co (Rockford, IL, USA). 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoyl) sphingosine (NBD-$C_6$-Cer) was from Life Technologies (Italy). D-threo1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were from (Biomol International, Plymouth Meeting, PA). Perifosine (D-21266 o octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate) was from Aeterna Zentaris (Frankfurt am Maim, Germany). PD98059 (2-(2-amino-3-methoxyphenyl)-chromone) was from Enzo Life Sciences (Farmingdale, NY, USA).

## METHODS

### 4.1 Cell cultures

U87MG, CCF-STTG1 and T98G human glioblastoma multiforme cell lines were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). U87MG cell line was cultured in a humidified atmosphere at 37° C with 5% $CO_2$, and maintained in DMEM containing: 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; CCF-STTG1 and T98G cell lines were cultured in DMEM supplemented with also 1 mM sodium pyruvate. The culture medium was usually changed every 48 hours. Cells were maintained by serial passages in 75 $cm^2$ culture flasks. At reaching confluence, subculture was prepared using a 0.025% trypsin–0.01% EDTA solution. Cells were re-suspended in DMEM 10% FCS, counted by trypan blue dye exclusion test using a Neubauer emocytometer, and then re-plated ($20\times10^3$ cells/$cm^2$) to allow cell propagation. All cell models were plated for experiments at the opportune density in different culture plate dishes (3.5, 6 or 10 cm of diameter) or in 24-well plates (1.8 cm of diameter).

### 4.2 Cell treatments

For cell treatments, stock solutions were prepared by dissolving the different molecules as follows:
- PF: 64.8 mM stock solutions were prepared in absolute ethanol;
- LY294002: 32.5 mM stock solutions were prepared in DMSO;
- PD98059: 50 stock solutions were prepared in DMSO;
- D609: 10 mM were prepared in distilled water;
- PPMP: 4 mM were prepared in absolute ethanol;
- PDMP: 10 mM were prepared in absolute ethanol;

Stock solutions were diluted extemporaneously in fresh medium at the desired concentrations and administered to cells for the indicated period of time. Of relevance, ethanol and DMSO final concentrations in the culture medium were always maintained below 0.5%, concentrations that do not affect cell survival. In each experiment untreated cells were incubated with vehicles at the same final concentration of treated cells as controls.

## 4.3 Analysis of cell viability: MTT assay

Cell viability was determined by MTT colorimetric assay. U87MG, CCF-STTG1 and T98G cells were seeded at 1 x $10^4$, 6.5 x $10^4$ and 1.8 x $10^4$ cells/$cm^2$ respectively in 24 well plates. The day after cells were treated with different agents for the indicated periods of time. The medium was then replaced by MTT dissolved in fresh medium (0.8 mg/ml) for 4 hours. The formazan crystals formed in viable cells were then solubilized in iso-propanol/formic acid (95:5 v/v) for 10 minutes with constant stirring to ameliorate the lysis process. The absorbance at 570 nm was measured using a microplate reader (Wallack Multilabel Counter, Perkin Elmer, Boston, MA, USA).

## 4.4 Immunoblotting analyses

Protein expression levels were evaluated using different conditions of cell lysis, SDS-PAGE and Western blotting depending on the antigen analysed, as described below.
In all cases a small aliquot of cell lysates was analyzed for the protein content with the Comassie Blue-based assay (Sedmak and Grossberg, 1977). The remaining lysate was denatured by the addition of Sample Buffer 4X (containing 0.25 M Tris- HCl pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 0.4 M DTT) and by heating for 5 minutes at 100 °C. Then, proteins were resolved by SDS-PAGE and detected by Western blotting as follows.

*Phosho-Akt*

Cells were washed with ice cold PBS for three times and lysed with a lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM $Na_3VO_4$, 10 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM PMSF in the presence of proteases inhibitors (2 µg/ml pepstatin, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). After 20 minutes of constant stirring at 4 °C, solubilized proteins were centrifuged at 8000 x g for 10 minutes at 4°C.
Proteins were resolved by SDS-PAGE on 10% of polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were then blocked, under constant stirring, for 1 hour at room temperature in Tris-buffered saline (25mM Tris-Cl pH 7.4, 154 mM NaCl), containing 0.1% Tween 20 and 5% skim milk. Once blocked, membranes were incubated overnight at 4 °C with a primary antibody against phospho-Akt (Ser473) (1:1000) and then with goat anti-mouse IgG (H+L) peroxidase-conjugated (1:2000) as a secondary antibody for 1 hour at room temperature.

*Phosho-ERK and total ERK*

Cells were washed with ice cold PBS containing 100 µM $Na_3VO_4$ for three times and lysed with a lysis buffer containing 20 mM Tris-HCl pH 7.4, 1.5 mM EGTA, 1% Triton X 100, 5 mM Na-β-glicerophosphate, 2 mM $Na_3VO_4$, 2 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM PMSF, 1 mM benzamidine, in the presence of proteases inhibitors (2 µg/ml aprotinin, and 2 µg/ml leupeptin). After 20 minutes of constant stirring at 4 °C, solubilized proteins were centrifuged at 8000 x g for 10 minutes at 4 °C. Cell lysates were homogenized using a probe type sonicator.
Proteins were resolved by SDS-PAGE on 10% of polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were then blocked, under constant stirring, for 1 hour at room temperature in Tris-buffered saline (25mM Tris-Cl pH 7.4, 154 mM NaCl), containing 0.1% Tween-20 and 5% skim milk. Once blocked, membranes were incubated for 1 h at room temperature with a primary antibody against phospho-ERK (pThr/pTyr) (1:500) and then with goat anti-rabbit IgG (H+L) peroxidase-conjugated (1:1350) as a secondary antibody for 1 hour at room temperature. The membranes were stripped 30 minutes at 50 °C in a buffer containing 2% SDS, 100 mM DTT, 0.5 M Tris-HCl pH 6.8, washed in TBS-T and blocked, under constant stirring, for 1 hour in Tris-buffered saline (25mM Tris-Cl pH 7.4, 154 mM NaCl) containing 0.1% Tween-20 (TBS-T) and 5% skim milk. Once blocked, membranes were incubated for 1 h at room temperature with a primary antibody against ERK2 (1:500) and then with goat anti-mouse IgG (H+L) peroxidase-conjugated (1:10000) as a secondary antibody for 1 hour at room temperature.

*LC3B*

Cells were washed with ice cold PBS for three times and lysed with a lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM $Na_3VO_4$, 10 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM EDTA, 1 mM PMSF in the presence of proteases inhibitors (2 µg/ml pepstatin, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). After 20 minutes of constant stirring at 4 °C, solubilized proteins were centrifuged at 8000 x g for 10 minutes at 4 °C.
Proteins were resolved by SDS-PAGE on 18% of polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were then blocked, under constant stirring, for 1 hour at room temperature in Tris-buffered saline (25mM Tris-Cl pH 7.4, 154 mM NaCl), containing 0.05% Tween-20 and 5% skim milk. Once blocked, membranes were incubated for 1 h at room temperature with a primary antibody against LC3B (1:1000) and then with goat anti-rabbit IgG (H+L) peroxidase-conjugated (1:1000) as a secondary antibody for 1 hour at room temperature.

In all cases, immunoblots were probed with an antibody for GAPDH as a protein loading control. Bound antibodies were visualized by the enhanced chemiluminescence method using SuperSignal West Pico Maximum sensitivity Chemioluminescent Substrate, and membranes were exposed to Kodak Biomax films. The relative abundance of each protein band was analysed via densitometry scanning of the exposed films.

## 4.5 Fluorescence studies: cell apoptosis analysis with Höechst staining and acidic vesicular organelles detection with acridine orange staining

U87MG, CCF-STTG1 and T98G cells were seeded at 1 x $10^4$ cell/$cm^2$, 6.5 x $10^4$ cell/$cm^2$, and 1.8 x $10^4$ cell/$cm^2$ respectively on a glass coverslip. After 48 hours, cells were incubated with PF at the indicated concentrations in culture medium. After 48 hours or 72 hours of treatment, cells were incubated in the dark at 37 °C with 1 µg/mL acridine orange (Sigma-Aldrich, St Louis, MO) for 20 min and with 5 µM Höechst 33342 (Sigma-Aldrich, St Louis, MO) in the last 15 min. Cells were then washed three times in PBS for 5 minutes with constant stirring, fixed with 0.5% glutaraldehyde solution in PBS for 10 minutes at 4°C, and analyzed with a fluorescence microscope (Olympus BX-50) equipped with a fast high-resolution CCD camera (Colorview 12) and an image analytical software (Soft Imaging System GmbH, Olympus, Munster, Germany).

## 4.6 Metabolic studies

Pulse experiments with [C3-$^3$H]Sphingosine

U87MG cells were seeded on 35 mm dishes in culture medium at 2.13 x $10^4$ cell/$cm^2$. After 48 hours, cells were incubated in fresh culture medium in the presence or absence of 10 µM PF for 4 hours. In the last 30 minutes or in the last 120 minutes of treatment, cells were pulsed with 0.5 µCi/dish radiolabeled sphingosine ([C3-$^3$H]Sphingosine, 21.4 Ci/mmole) in the conditioned medium. Cells were then collected and submitted to lipid extraction, partitioning, and methanolysis, as described below.

Pulse experiments with [methyl-3H]Choline

U87MG cells were seeded on 35 mm dishes in culture medium at 2.13 x $10^4$ cell/$cm^2$. After 48 hours, medium was gently removed and cells were then fed with 1.92 µCi/dish radiolabeled choline ([methyl-3H]Choline, 75 Ci/mmole), in fresh culture medium in presence or absence of 10 µM PF for 4 hours. Cells were then collected and submitted to lipid extraction and partitioning, as described below.

Extraction and partitioning of intracellular lipids

At the end of pulse, cells were washed three times in ice-cold PBS and harvested with an appropriate volume of methanol. Then, an appropriate volume of chloroform was added in order to obtain the final chloroform/methanol ratio 2:1 (v/v). After mixing for 15 minutes, samples were centrifuged at 10000 x g for 15 minutes at 4 °C. The supernatant was collected and the pellet was re-extracted by adding 500 µl chloroform/methanol 2:1 (v/v), mixed and centrifuged. The second supernatant obtained was added to the first one. The two supernatants combined represent the total lipid extract (TLE). An aliquot of the TLE was counted for radioactivity by liquid scintillation. Pellets obtained after lipid extraction, containing cellular proteins, were dried a under nitrogen stream and digested over night with 50 µl 1N NaOH at R.T. Digestion was stopped with 450 µl distilled water. Aliquots were analyzed for protein content with the Lowry based assay (Lowry et al., 1951). An aliquot of 1.5 ml of TLE was partitioned by adding 375 µl of distilled water. After vigorous and repeated shaking, phases were separated by centrifugation at 8500 x g for 5 minutes at 4°C. At the end of the centrifugation a biphasic solution was obtained, composed of an upper aqueous phase, containing the more polar intracellular lipids (S1P and gangliosides) and a lower organic phase, containing the more apolar intracellular lipids (Cer, GlcCer , LacCer, Sph, SM and glycerophospholipids). Aqueous phase was transferred to new glass tube, while organic phase was washed with a volume of chloroform/methanol /water 3:48:47 (v:v:v) corresponding to that of the aqueous phase collected, mixed and centrifuged. The second aqueous phase obtained was added to the first one. Organic phase was evaporated under a nitrogen stream, dissolved in chloroform/methanol 2:1 (v/v) counted for radioactivity by liquid scintillation and then analysed by HPTLC (as described below). Alternatively, organic phase was submitted to mild alkaline methanolysis in order to remove the glycerophospholipids.

Mild alkaline methanolysis of the organic phase

The organic phase was evaporated to dryness under a nitrogen stream and then re-dissolved in 50 µl chloroform. After the addition of 50 µl of 0.2 N KOH in methanol, the samples were mixed and incubated 1 hour at 37 °C under constant stirring. The reaction was stopped by neutralization with 60 µl of 0.2 N HCl in methanol. Then, 90 µl methanol and 350 µl chloroform were added in order to reach the final chloroform/methanol ratio 2:1 (v/v). After mixing, phases were separated by the addition of 95 µl water and centrifugation at 8500 x g for 5 minutes at 4 °C. The upper aqueous phase was removed and the lower organic phase was dried under a nitrogen stream, re-suspended with 300 µl chloroform/methanol 2:1 (v/v), counted for radioactivity by liquid scintillation and analysed by HPTLC (as described below).

Separation and quantification of radiolabeled sphingolipids by HPTLC

The radiolabeled sphingolipids recovered in the organic phase or in the methanolysed organic phase were submitted to high performance thin layer chromatography (HPTLC) on silica gel plates, developed in a chromatographic chamber by the use of appropriate solvent systems as follows.
Sphingolipids contained in the methanolysed organic phase from cells pulsed with [C3-$^{3}$H]Sphingosine were separated using chloroform/methanol/water 110:40:6 (v/v/v) as solvent system. A standard mix composed of tritiated Cer, GlcCer, LacCer, Sph, and SM was chromatographed on the same plate and used as internal standard.
Phospholipids contained in the organic phase from cells pulsed with [methyl-$^{3}$H]Choline were separated using chloroform/methanol/acetic acid/water 50:30:8:5 (v/v/v/v) as solvent system. Radiolabeled SM and PC were chromatographed on the same plate and used as internal standard.
HPTLC plates were then dried and submitted to digital autoradiography (Beta-Imager 2000, Biospace, Paris, FR). The radioactivity associated with individual lipids was quantified by the beta vision analysis software (Biospace, Paris, FR).

## 4.7 Sphingomyelin synthase and glucosylceramide synthase activity assays

*Sphingomyelin synthase and glucosylceramide synthase activity assay in cell culture*

U87MG cells were seeded on 60 mm dishes in culture medium at 2.13 x $10^{4}$ cell/cm$^{2}$. After 48 hours, cells were incubated with or without 10 µM PF for 3 hours and 30 minutes in culture

medium. At the end of the treatments, the medium was collected and the cells were loaded with 5 μM NBD-$C_6$-Cer (as 1:1 complex with fatty acid free BSA) in KRH solution, containing 25 mM HEPES-NaOH pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 6 mM glucose and 2 mM $CaCl_2$, at 4 °C for 30 minutes. After loading, the cells were washed twice in PBS containing fatty acid free BSA (0.34 mg/ml) and then incubated for 30 minutes at 37 °C in the appropriate conditioned medium. At the end of the incubation, cells were immediately put at 4 °C to stop the enzymatic reaction; then, cell lipids were extracted with chloroform/methanol as described above in order to obtain the TLE. A small aliquot of TLE from each sample was analyzed for fluorescence-labeled lipids content with a luminescence spectrometer (LS50B PerkinElmer). Lipids were separated by high performance thin-layer chromatography (HPTLC) using chloroform/methanol/15 mM $CaCl_2$ 60:35:8 (vol/vol/vol) as solvent system. The bands corresponding to the reaction products NBD-$C_6$-SM and NBD-$C_6$-GlcCer and to the substrate NBD-$C_6$-Cer of each sample, were separately scraped off the HPTLC plates and lipids were extracted from the silica gel with 500 μl chloroform/methanol/0.1 M KCl 1:2:0.8 (vol/vol/vol). After mixing, samples were centrifuged at 10000 x g for 15 minutes at 4 °C. The supernatant was collected and the precipitated was re-extracted with 500 μl chloroform/methanol/0.1 M KCl 1:2:0.8 (vol/vol/vol), mixed and centrifuged. The second supernatant obtained was added to the first one. Fluorescence-labeled SM, GlcCer and Cer were quantified with a luminescence spectrometer (LS50B PerkinElmer).

*Cell-free sphingomyelin synthase and glucosylceramide synthase activity assay*

U87MG cells were seeded on 100 mm dishes in culture medium at 2.13 x $10^4$ cell/$cm^2$. After 48 hours, cells were washed three times with ice-cold PBS, harvested and homogenized using a probe type sonicator. Protein concentration of cell homogenates was determined by the Lowry method (Lowry et al., 1951). SMS and GCS activities were assayed using experimental conditions known to selectively favor SMS or GCS activity.

For the *SMS activity assay*, a reaction mixtures consisting of 50 mM Tris-HCl pH 7.4, 25 mM KCl, 0.5 mM EDTA, and 40 μM NBD-$C_6$-Ceramide (as 1:1 complex with fatty acid free BSA) supplemented with or without 10 μM PF was prepared and added to 12.5 γ or 25 γ of cell homogenate in a final volume of 50 μl.

For the *GCS activity assay*, a reaction mixtures consisting of 50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM UDP-glucose, 10 mM $MnCl_2$, and 40 μM NBD-$C_6$-Ceramide (as 1:1 complex with fatty acid

free BSA) supplemented with or without 10 µM PF was prepared and added to 12.5 γ or 25 γ of cell homogenate in a final volume of 50 µl.

After an incubation period of 15 minutes at 37 °C in the dark and in constant stirring, the reaction was stopped by adding 150 µl chloroform/methanol 1:2 (v/v).

After mixing, samples were centrifuged at 10000 x g for 10 minutes at 4 °C. After being transferred to new glass tube, 150 µl chloroform was added to the supernatant and then the samples were mixed and centrifuged at 8500 x g for 10 minutes at 4 °C. At the end of the centrifugation a biphasic solution was obtained, composed of an upper aqueous phase and a lower organic phase, containing the reaction products NBD-$C_6$-SM and NBD-$C_6$-GlcCer and the remaining substrate NBD-$C_6$-Cer. The organic phase was transferred to a new glass tube, dried under nitrogen stream and resuspended in chloroform/methanol 2:1 (v/v). Fluorescence-labeled SM, GlcCer, and Cer were then resolved on HPTLC, scraped off the HPTLC plates, extracted from the silica gel, and quantified with a luminescence spectrometer (LS50B PerkinElmer) as describe above.

## 4.8 Analysis of the endogenous phospholipids and gangliosides

U87MG cells were seeded on 100 mm dishes in culture medium at 2.13 x $10^4$ cell/$cm^2$. After 48 hours, cells were incubated with or without 10 µM PF in fresh culture medium supplemented with 0.5% FCS for 48 hours. At the end of treatments, cell lipids were extracted with chloroform/methanol (as described above) in order to obtain the TLE and then partitioned (as described above). An aliquot of the organic phase containing phospholipids as lipid molecules of interest was used to determine the phospholipid content, according to the Bartlett assay, a colorimetric assay based on the determination of liberated inorganic phosphate (Bartlett, 1959). Phospholipids were submitted to HPTLC using chloroform/methanol/acetic acid/water 50:30:8:5 (v/v/v/v) as a solvent system. The HPTLC plate was then sprayed with the Vaskovsky V.E. and Kostetsky E.Y. reagent (Vaskovsky and Kostetsky, 1968) and incubated at 120 °C for 15 minutes. The relative abundance of each spot was quantified by densitometric scanning of the HPTLC plate.

The acqueous phase containing gangliosides as lipid molecules of interest was dried under a nitrogen stream, and resuspended in chloroform/methanol 2:1 (v/v). Gangliosides were then submitted to HPTLC using chloroform/methanol/15 mM $CaCl_2$ 55:45:10 (vol/vol/vol) as a solvent system. The HPTLC plate was then sprayed with the Ehrlich reagent and incubated at 120 °C for 15 minutes. The relative abundance of each spot was quantified by densitometric scanning of the HPTLC plate.

## 4.9 Protein assays

Protein content was evaluated using the Bradford/Comassie blue-based assay (Sedmak and Grossberg, 1977) or the Lowry assay (Lowry et al., 1951). In both cases bovine serum albumin fraction V was used as standard. The protein content was obtained by spectrophotometric reading at 595 nm for the Bradford assay and 750 nm for the Lowry assay.

## 4.10 Statistical analysis

Results are expressed as mean value ± SD for at least three independent experiments. The statistical significance of differences was determined by the Student's t test. A p value $< 0.05$ was considered significant. Data were analyzed using StatMate software, version 4.0 (GraphPad).

# RESULTS

## 5.1 Effect of Perifosine on GBM cells viability

Evidence in literature indicates that the cytotoxic effects of PF can differ depending on cell types (van Blitterswijk and Verheij, 2008). Up to now, only few studies have investigated the efficacy of PF against gliomas (Becher et al., 2010; de la Pena et al., 2006; Momota et al., 2005; Pitter et al., 2011; Qin et al., 2013; Rios-Marco et al., 2013; Rios-Marco et al., 2015) and much remains to be clarified about its mechanisms of action. So, we first evaluated the effect of PF on cell survival in three human GBM cell lines: T98G, CCF-STTG1 and U87MG, which are characterized by a different mutation status of PTEN. In particular, T98G cells are wild type for PTEN, while CCF-STTG1 and U87MG cells are mutant for PTEN (Zhang et al., 2007b ; ours unpublished data) and therefore have constitutively activated Akt. For this purpose, the three cell lines were treated with PF at concentrations ranging from 5 µM to 60 µM in complete medium. After 48 hours we performed MTT assay to evaluate cell viability. The results obtained demonstrated that PF reduced cell viability in a dose-dependent manner in all GBM cell lines tested (Fig. 6). Moreover, our experiments show that this cytotoxic effects increased with increasing exposure time (data not shown). The drug sensitivity parameter $ED_{50}$ (dose required to reduce cell viability by 50%) was calculated after 48 hours of treatment with PF. The T98G, CCF-STTG1, and U87MG cell lines showed an $ED_{50}$ value of 12.9 ± 0.65 µM, 37.3 ± 1.86 µM, and 19.3 ± 0.96 µM, respectively. These results suggest that in these cell lines the sensitivity to PF did not correlate directly with basal levels of p-Akt and PF exerts its effects on cell survival independently of PTEN mutation status.

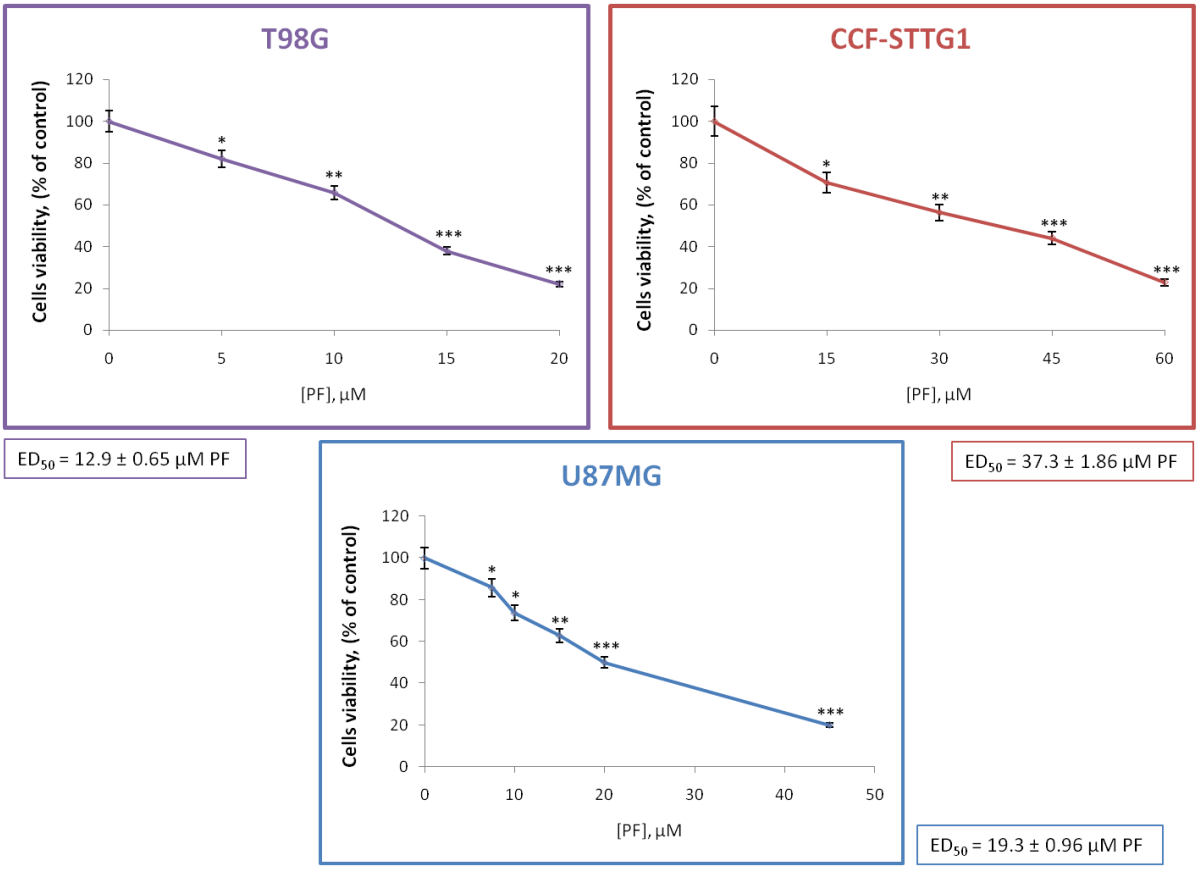


**Figure 6.** *PF cytotoxicity in different GBM cell lines.* Cells were left untreated or exposed to different doses of PF. Cells viability was assessed after 48 h of treatment by MTT assay. The results are expressed as a percentage of viable cells with respect to control untreated cells and presented as means ± SD of three independent experiments. Asterisks represent statistical significance determined by Student's t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs untreated cells).

## 5.2 Effect of Perifosine on Akt activation in GBM cells

Subsequently, we investigated the effect of PF on Akt phosphorylation. We treated the three GBM cell lines with concentrations of PF giving a 25-40% decrease of cell viability in complete medium supplemented with fetal bovine serum as growth stimulus. Moreover, cells were treated with LY294002 (LY29), a known PI3K/Akt inhibitor (Knight, 2010), as positive control. At the indicated times, cells were lysed and the same amount of proteins for each sample was resolved in SDS-PAGE. Our immunoblotting analyses (reported in Fig. 7, panel A and B) indicated that PF promoted a decrease of p-Akt(Ser473) levels in a time-dependent manner, with a similar trend in all GBM cell lines analyzed. We found that PF reduced the p-Akt levels in T98G and CCF-STTG1 cells by 50% and 60% respectively after 3 hours of treatment, reaching an almost total inhibition of Akt after 24 hours of treatment (Fig. 7, panel A). In U87MG cells 10 µM PF induced a decrease of p-Akt levels of 30% and 70% after 4 and 24 hours of treatment respectively, while 20 µM PF induced a decrease of p-Akt levels of about 30% and 70% after only 2 and 4 hours of treatment

respectively, and induced an almost complete inhibition of Akt already after 8 hours of treatment (Fig. 7, panel B). On the other hand, in the same GBM cell lines, p-Akt was undetectable after just 1 hour of treatment with LY29 (Fig. 7, panel A and B).

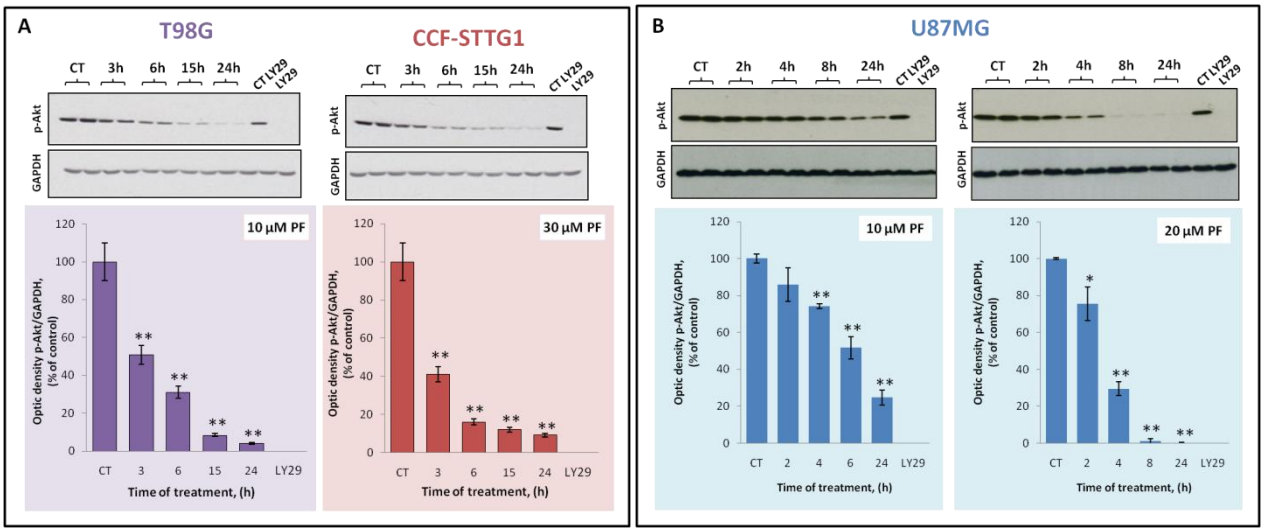


**Figure 7.** *Effect of PF on Akt phosphorylation in different GBM cells.* Cells were treated in the presence or absence of PF at the indicated concentrations and for the indicated times or in the presence or absence of 10 µM LY29 for 1 h. Cells were then harvested in lysis buffer for immunoblotting analysis of p-Akt and GAPDH levels, as described in "Material and Methods" section. Equal amounts of protein from each sample were analyzed by immunoblotting with an anti-p-Akt antibody and an anti-GAPDH antibody. The levels of p-Akt was determined by densitometric quantitation and normalized for GAPDH levels. Values are mean ± SD of three independent experiments. Asterisks represent significant differences between control and treated cells (*p <0.5; **p < 0.01, Student's *t* test).

## 5.3 Effect of Perifosine in combination with LY294002 on U87MG cells viability

As previously mentioned, the inhibition of Akt activation/phosphorylation is considered one of the main mechanism by which PF causes its cytotoxic effect (Gills and Dennis, 2009; Hennessy et al., 2007; Kondapaka et al., 2003; van Blitterswijk and Verheij, 2013). However, recently it has been reported that PF could determine also Akt-independent effects (Fensterle et al., 2014), resulting in cell demise. So, we wonder if PF is still able to induce cytotoxic effects even in a condition in which Akt is completely inhibited, such as treating cells with the LY29 compound. Therefore, U87MG cells were treated with PF in presence or absence of LY29. After 48-72 hours cell viability was evaluated by MTT assay. As shown in Fig. 8, both PF and LY29 alone were able to decrease cell viability. Moreover, the co-treatment of PF and 5 µM LY29 induced an about 24% and 30% decrease of cell viability at 48 and 72 hours respectively, compared to cells treated only with 5 µM LY29. Similar results were obtained using 10 µM LY29. This result thus suggested that PF exerts its cytotoxic effect through mechanisms more than just blocking Akt.

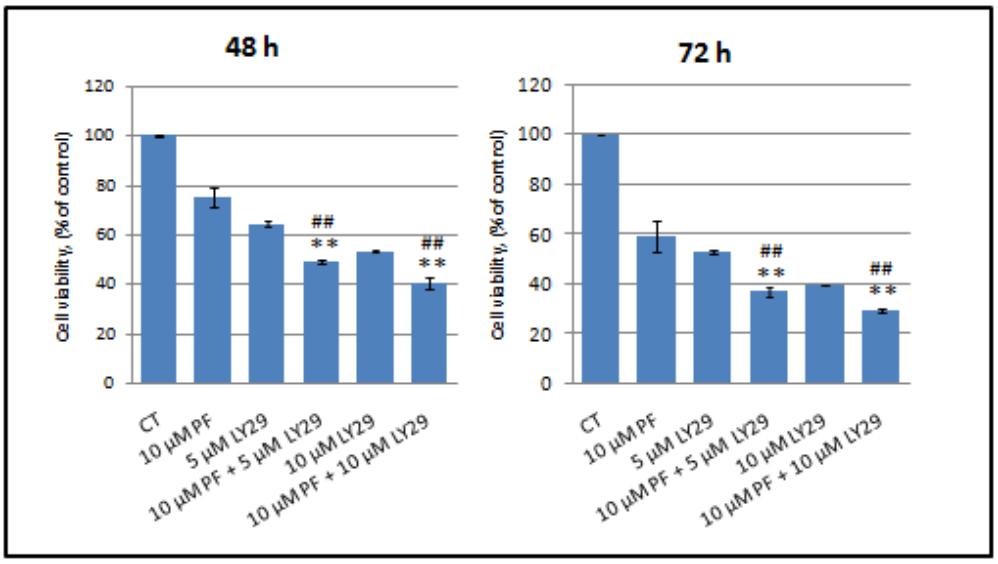


**Figure 8.** *Effect of PF in combination with LY29 on U87MG cells viability.* Cells were cultured in control media or exposed to 10 µM PF, 10 µM LY29, separately or in combination, as indicated. Cells viability was assessed after 48 h and 72 h of treatment by MTT assay. The results are expressed as a percentage of viable cells with respect to control untreated cells and presented as means ± SD of three independent experiments. Asterisks represent statistical significance determined by Student's t test (*p < 0.05; **p < 0.01 vs PF-treated cells; $^{\#}$p<0.05; $^{\#\#}$p<0.01 vs LY29-treated cells).

## 5.4 Effect of Perifosine on [methyl-$^3$H]Choline metabolism in U87MG cells

Increasing evidence suggests that in different cell lines PF, such as other ALPs, is able to inhibit the rate-limiting enzyme CT and, consequently, the biosynthesis of PC (Marco et al., 2009; Tafesse et al., 2006; van Blitterswijk and Verheij, 2013; Wieder et al., 1998). In some cases, the inhibition of PC biosynthesis is considered a crucial event to lead to cell death, through still unclear mechanisms. PC is the donor of phosphocholine in the reaction catalyzed by the enzyme SMS (Diringer et al., 1972; Riboni et al., 1997). So, in accordance with the precursor-product relationship, the inhibition of the PC biosynthesis may also affect the SM biosynthesis from PC and Cer and, as a consequence, may induce the accumulation of this last pro-death lipid.

Based on these assumptions, we perform a pulse experiment with radiolabeled choline. Cells were fed with [methyl-$^3$H]Choline for 4 hours in the absence or presence of PF. We found that the uptake of radiolabeled choline was similar in treated and untreated cells (data not shown). On the other hand, PF treatment significantly reduced the incorporation of [methyl-$^3$H]Choline into PC and SM by 40 ± 3 % and 39 ± 5.2 % respectively, compared to control cells. Of note, PF treatment does not affect the [$^3$H]PC/[$^3$H]SM ratio with respect to untreated cells, suggesting that even if the PC biosynthesis was reduced, it was not a limiting factor for SM synthesis (Fig. 9).

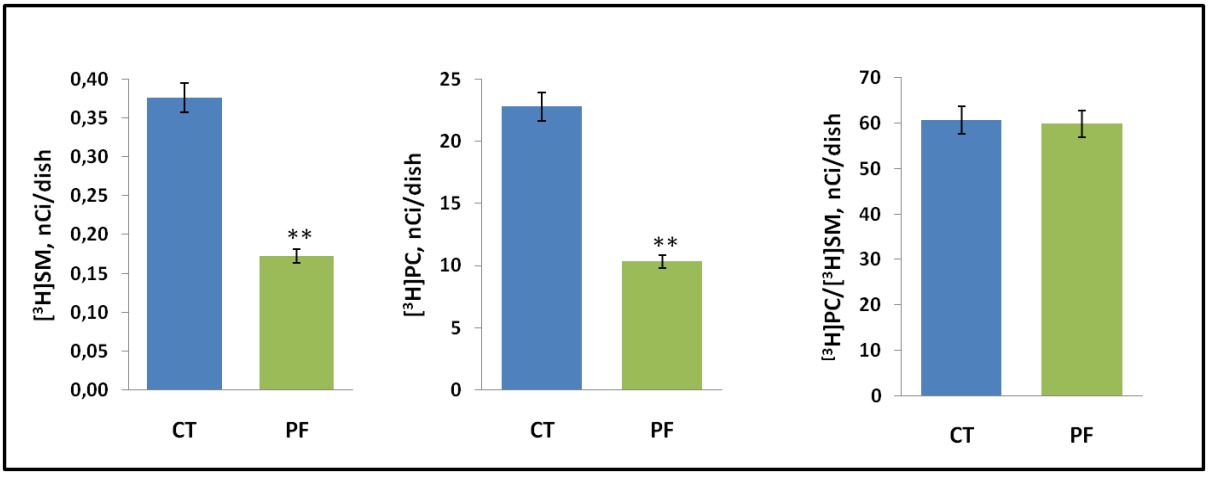


**Figure 9.** *[methyl-$^3$H]Choline metabolism in 87MG cells treated with PF.* Cells were pulsed with 1.6 µCi/dish [methyl-$^3$H]Choline for 4 h in the absence or presence of 10 µM PF. At the end of pulse, cells were harvested and submitted to lipid extraction and partitioning. The organic phase was analysed by HPTLC and submitted to digital autoradiography. Experiments were done in triplicate, data are mean ± SD. Asterisks represent significant differences between control and treated cells (*p < 0.5; **p < 0.01, Student's *t* test).

## 5.5 Effect of Perifosine on [C3-$^3$H]Sphingosine metabolism in U87MG cells

Since we demonstrated that PF affected SM biosynthesis in U87MG cells, we investigated the effect of PF on Cer metabolism. To this purpose, we performed short time pulse experiments (30 and 120 minutes), in the absence or presence of PF, by feeding cells with [C3-$^3$H]Sphingosine. Cells were able to efficiently and rapidly internalize this radiolabelled SL, as Cer precursor. The incorporated radioactivity, present in the total lipid extract, increased in a time-dependent manner and was similar in untreated and treated cells (data not shown), indicating that treatment with PF did not alter the [C3-$^3$H]Sphingosine uptake. Moreover, at both 30 and 120 minutes of pulse, [C3-$^3$H]Sphingosine represented only a minor part of incorporated radioactivity in control and PF-treated cells (Fig. 10), suggesting that cells rapidly metabolized Sph in all considered experimental conditions tested (Fig. 10). More in detail, at short pulse times, most of the incorporated [C3-$^3$H]Sphingosine was metabolized to N-acylated compounds (constituted for the most part of Cer, and in a lesser extent, SM and GSLs). The total amount of N-acylation increased with pulse time and was very similar in control and PF-treated cells. Furthermore, cells were characterized by a time dependent increase in the levels of Cer metabolism products: [$^3$H]SM, [$^3$H]GlcCer and [$^3$H]LacCer. Of relevance, PF-treatment was able to significantly modify the distribution of the radioactivity associated to the different Sph N-acylated metabolites. In fact, [$^3$H]Cer level increased in a time-dependent manner after PF treatment, accounting for an increase of 10.6 ± 2.4 % and 32 ± 5.9 % at 30 minutes and 120 minutes pulse, respectively, in comparison with control cells. In parallel, the radioactivity associated to SM was reduced by 37 ± 8.4 % and 41 ± 2.6 % at 30 and

120 minutes pulse, respectively (Fig. 10). Furthermore, the radioactivity associated to GSLs (GlcCer + LacCer) were reduced by 16 ± 4.3 % and 16 ± 4.8 % at 30 and 120 minutes pulse, respectively in treated cells compared to control.

Therefore, our data indicate that in U87MG cells PF treatment increased Cer levels mostly as a result of diminished SM synthesis and, apparently, also as a result of diminished GSLs synthesis, without altering the process of Cer synthesis, evaluated from the N-acylation capacity of control and PF treated cells.

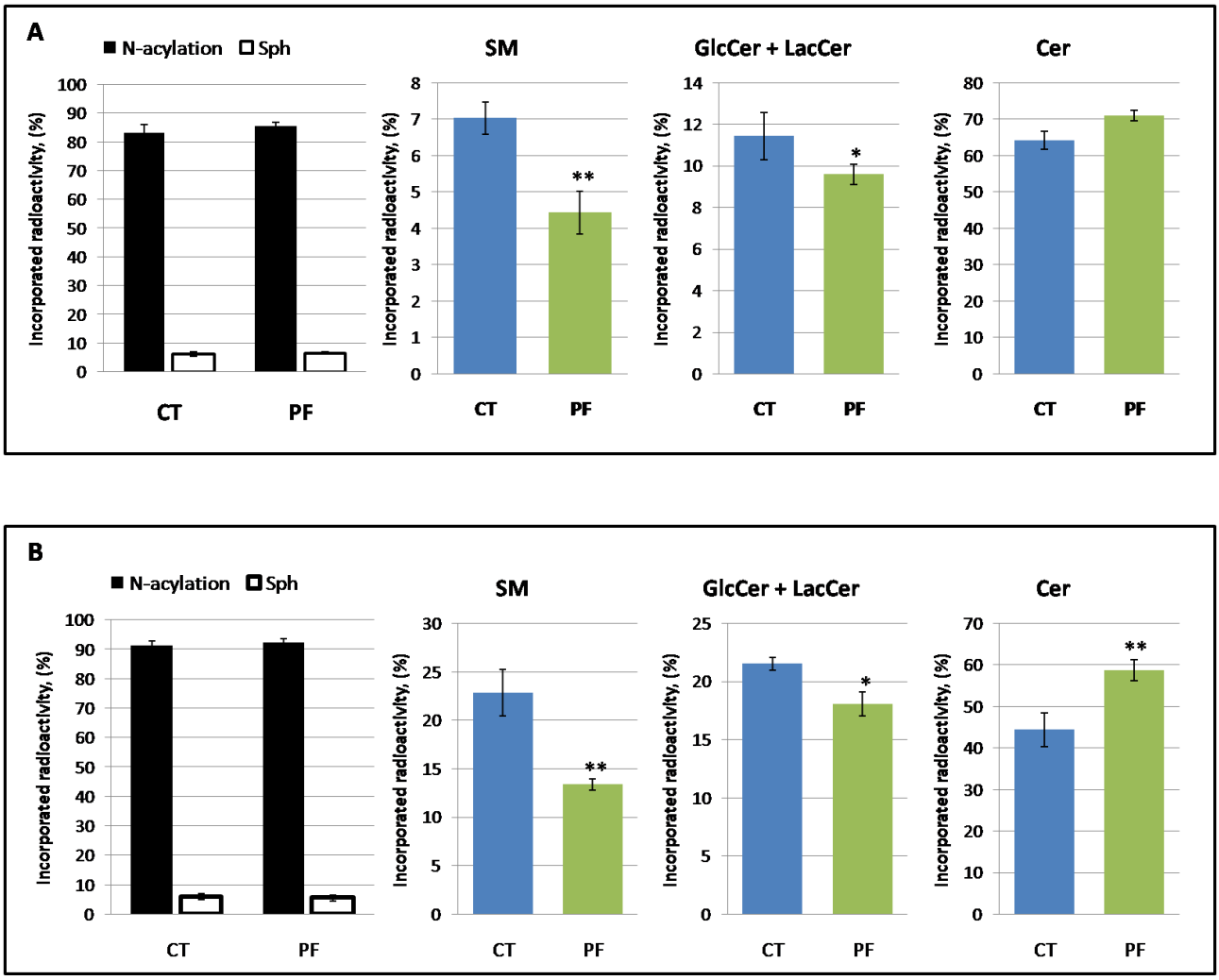


**Figure 10.** *[C3-$^{3}$H]Sphingosine metabolism in U87MG cells treated with PF.* Cells were incubated in complete medium in the presence or absence of 10 µM PF for 4 h. In the last 30 min **(A)** or in the last 120 min **(B)** of treatment, cells were pulsed with 0.5 µCi/dish [C3-$^{3}$H]Sphingosine in the conditioned medium. At the end of pulse, cells were harvested and submitted to lipid extraction and partitioning. The methanolized organic phase was analyzed by HPTLC and submitted to digital autoradiography. Radioactivity associated to the process of Sph N-acylation and intracellular unmetabolized [C3-$^{3}$H]Sphingosine is shown. Data are expressed as % of incorporated radioactivity. Values are the means ± SD of three independent experiments. Asterisks represent significant differences between control and treated cells (*$p < 0.5$; **$p < 0.01$, Student's *t* test).

## 5.6 Effects of Perifosine on sphingomyelin synthase and glucosylceramide synthase activity in U87MG cells

To better investigate the effects of PF treatment on Cer utilization by U87MG cells, we evaluated if PF is able to affect SMS and GCS activity.

To this purpose, we performed a SMS and GCS activity assay both in cell culture and cell-free, using a fluorescently labeled Cer derivative, NBD-$C_6$-Cer, as an efficient substrate for both SMS and GCS. In the enzymatic activity assay in cell culture, cells treated with or without PF were incubated with NBD-$C_6$-Cer. NBD-$C_6$-Cer has a high cellular permeability and, once internalized by cells, rapidly targets the Golgi, where it is utilized for the synthesis of NBD-$C_6$-SM and NBD-$C_6$-GlcCer (Chazotte, 2012). The results obtained show (Fig. 11, panel A) that the NBD-$C_6$-SM levels were reduced by about 23% in PF treated cells with respect to control, while the NBD-$C_6$-GlcCer levels were very similar in treated and untreated cells. In parallel, NBD-$C_6$-Cer levels were about 16% higher in PF treated cells than in the control. In the cell-free enzymatic activity assay, cellular homogenates were incubated with NBD-$C_6$-Cer in the presence or absence of PF with the aim to evaluate if PF was able to directly interacts with SMS and/or GCS activity. The cell-free enzymatic activity assay demonstrated that the activity of SMS and GCS was 27 ± 1.5 and 146 ± 24 nmol/mg protein/min, respectively in untreated control cells. In presence of PF any significant variation was observed with respect to untreated cells (Fig. 11, panel B). Taken together, these results indicate that PF affects SMS activity, but not GCS activity in U87MG cells and that this effect is due to a mechanism different from the direct interaction of PF with the enzyme.

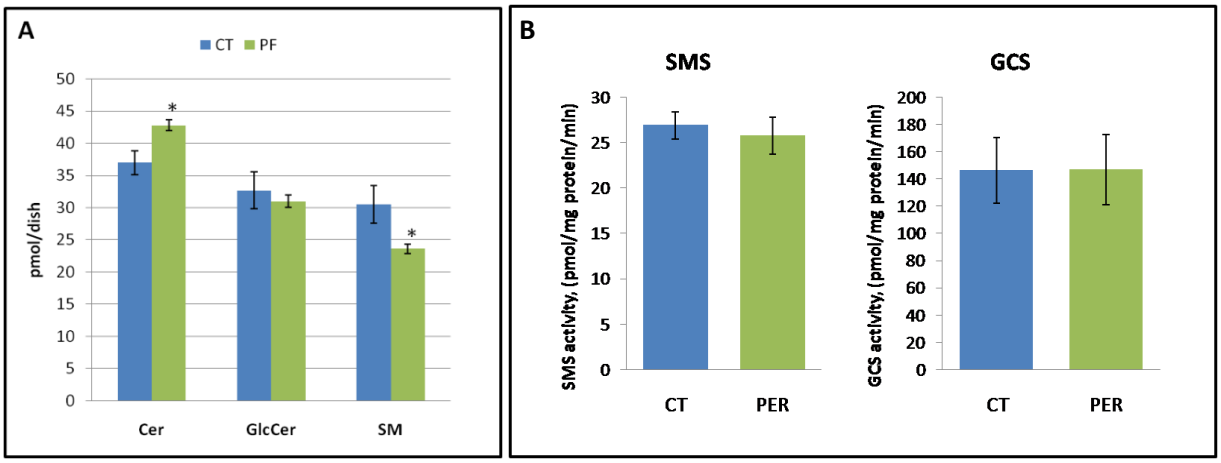


**Figure 11.** *Effect of PF on SMS and GCS activity in U87MG cells.* **(A)** SMS and GCS activity assay in cell culture. **(B)** Cell-free SMS and GCS activity assay. Experiments were done in triplicate, data are mean ± SD. Asterisks represent significant differences between control and treated cells (*p < 0.5; **p < 0.01, Student's *t* test).

## 5.7 Effects of Perifosine on endogenous level of Phospholipids in U87MG cells

Since we demonstrated that in U87MG cells PF is able to affect PC and SM biosynthesis, we evaluated if PF could affect also the total amount of endogenous PC and SM and if it changes cell phospholipids (PLs) profile. We found (Fig.12) that the total PLs/protein ratio was reduced by about 26% in treated cells with respect to control (from 488 ± 3.1 nmol PLs/mg protein to 362 ± 20.6 nmol PLs/mg protein) and that treated cell exhibited a different PLs pattern. Indeed, cells exposed to PF were poorer in PC (-45%), SM (-32%), and PS (-16%), and richer in PE (+29%) than control cells.

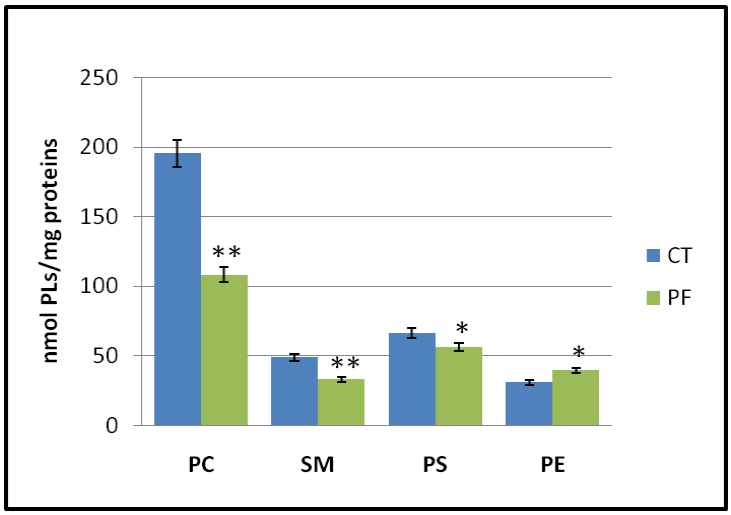


**Figure 12.** *Effect of PF on endogenous levels of Phospholipids in U87MG cells.* Cells were treated in the absence or presence of 7 µM PF in complete medium supplemented with 0.5% FCS for 48 h and then harvested and submitted to lipid extraction and partitioning as described in "Material and Methods" section. The organic phase was analyzed by HPTLC and PLs were visualized with the Vaskovsky V.E. and Kostetsky E.Y. reagent. Experiments were done in triplicate, data are mean ± SD. Asterisks represent significant differences between control and treated cells (*$p < 0.5$; **$p < 0.01$, Student's t test).

## 5.8 Effects of Perifosine on endogenous level of Gangliosides in U87MG cells

Since PF slightly but significantly decrease GlcCer and LacCer levels, as we demonstrated in metabolic studies performed with [C3-$^3$H]Sphingosine, without affect GCS activity, as we determined in *in vitro* and *in silico* enzymatic assays, we investigate if the reduction in the amount of GlcCer and LacCer observed could be due to their rapid utilization for the synthesis of more complex GSLs, such as gangliosides. To asses this hypothesis, we analyze if PF is able to alter the endogenous levels of gangliosides in U87MG cells. As shown in Fig. 13, PF was able to induce a 60 ± 3 % increase in endogenous gangliosides levels, compared to untreated cells.

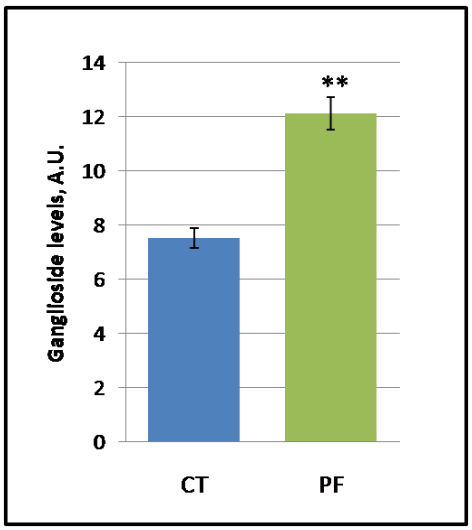


**Figure 13.** *Effect of PF on endogenous level of gangliosides in U87MG cells.* Cells were treated in absence or presence of 7 µM PF in complete medium supplemented with 0.5% FCS for 48 hours and then harvested and submitted to lipid extraction and partitioning as described in "Material and Methods" section. Gangliosides present in the aqueous phase were analyzed by HPTLC and then visualized with the Ehrlich reagent. Experiments were done in triplicate, data are mean ± SD. Asterisks represent significant differences between control and treated cells (*$p < 0.5$;**$p < 0.01$, Student's t test).

## 5.9 Analysis of the mechanism of Perifosine-induced cells death

It has been reported that PF can induce both apoptosis and/or autophagy, depending on cell type (Fensterle et al., 2014; Fu et al., 2009; Qin et al., 2013; van Blitterswijk and Verheij, 2013). To assess whether PF-exposed GBM cell lines undergo apoptosis and/or autophagy, we performed both Höechst 33342 and acridine orange (AO) cell staining after 48 hours of treatment with PF at concentration giving 25-40% decrease of cell viability. The first type of staining allows highlighting a mechanism of cell death by apoptosis, while the second one allows detecting the presence of autophagic cells. As shown in Fig. 14, in all the three GBM cell lines analyzed, Höechst 33342 staining showed neither condensed chromatin, nor any fragmented nuclei in PF-treated cells, thus indicating the absence of apoptosis; on the other hand, AO staining showed the formation of a discrete number of cytoplasmatic spots emitting red-orange fluorescence, representative of acidic vesicular organelles, that are absent in non-treated cells, suggesting that PF induced autophagy. Even after 72 hours of PF-treatment, signs of apoptosis were undetectable in U87MG cells, while the number of acidic vesicular organelles augmented significantly (Fig. 14, panel C).

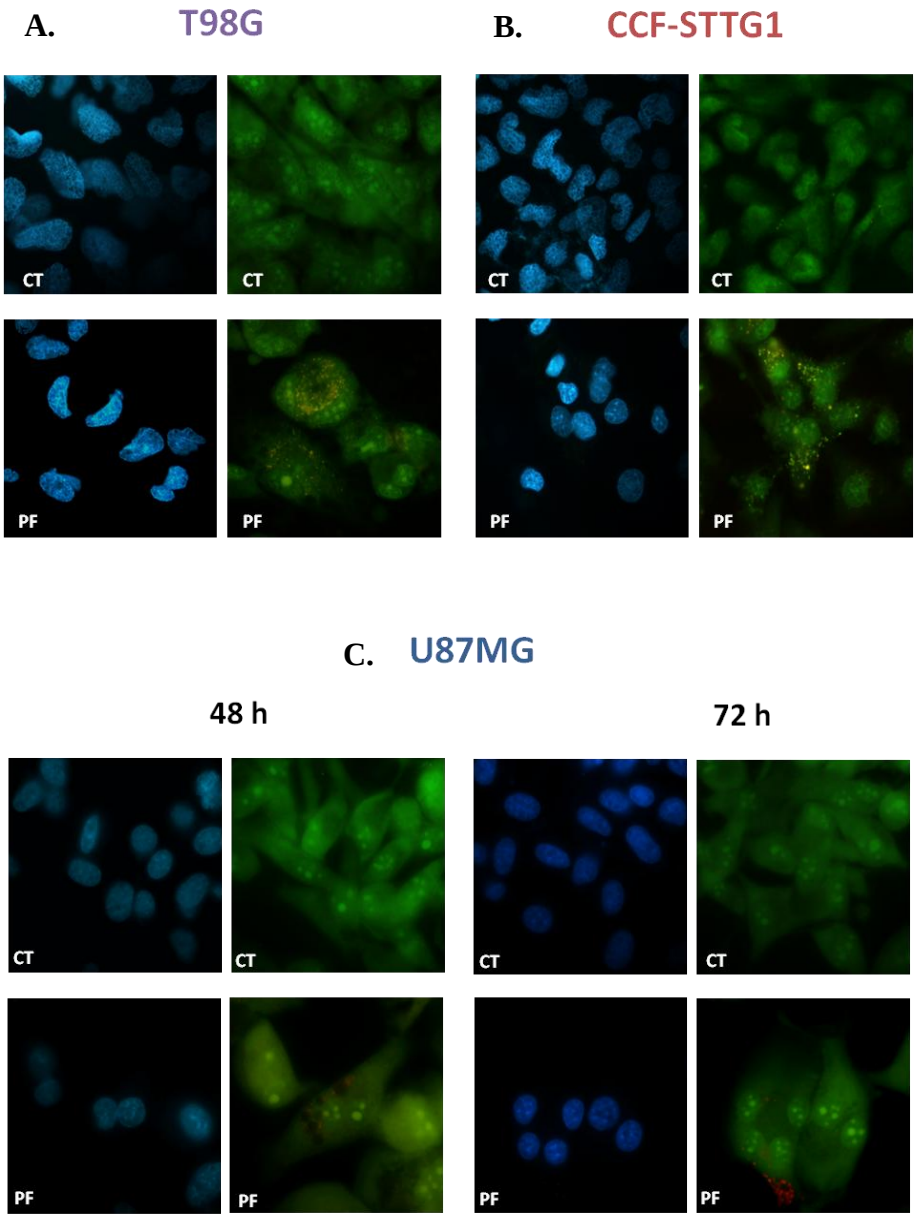


**Figure 14.** *Mechanism of PF-induced cell death.* GBMs cells grown on a glass coverslip were treated in absence or presence of (**A** and **C**) 10 µM, (**B**) 30 µM PF for 48 h and 72 h (**C**). Vital staining was performed labeling cells with 5 µM Höechst 33342 for 15 min and with 2 µM acridine orange for 20 min. Cells were then fixed and processed for immunofluorescence microscopy. The fluorescent microscopy images (magnification, 100X) were processed and printed identically.

It has been reported that the AO stain is not a selective marker of only autolysosomes, but can also accumulate in other intracellular acidic compartments (Robbins and Marcus, 1963). Different specific markers to monitor autophagy have been identified. Autophagosomes formation involves the localization of the protein LC3 (Microtubule-assocociated light chain protein 3) in autophagosomes, and, between the different homologs, LC3B is most commonly used for autophagy assays. In particular, it has been shown that two different isoforms of LC3B exist: LC3B-I, which is the cytoplasmic form of LC3B, and that corresponding to LC3B-II, which is the

preautophagosomal and autophagosomal membrane-bound form of LC3B. LC3B-II is characterized by the addition of phosphatidylethanolamine (PE) which it is essential for membrane binding. This addition induces changes in LC3B-II electrophoretic mobility during SDS-PAGE, LC3B-II migrating faster than LC3B-I while having a greater molecular weight. Hence, the amount of LC3B-II is closely correlated with the number of autophagosomes and it is considered as an excellent marker for autophagosomes detection (Mizushima and Yoshimori, 2007).

So, the presence of autophagy in U87MG cells exposed to PF was also evaluated analyzing by immunoblotting the LC3B conversion from LC3B-I to LC3B-II.

As shown in Fig. 15, in U87MG cells PF significantly increased the amount of LC3B-II protein in a time-dependent manner compared to untreated cells, confirming that PF induced autophagy.

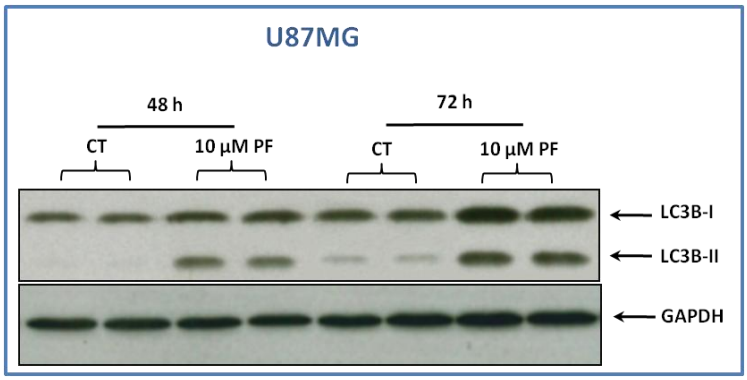


**Figure 15.** *Effect of PF on LC3B-II amount in U87MG cells.* Cells were treated with or without 10 µM PF for 48 and 72 h and then harvested in lysis buffer for immunoblotting analysis of LC3B-I, LC3B-II, and GAPDH levels, as described in "Material and Methods" section. Equal amounts of protein from each sample were analyzed by immunoblotting with an anti-LC3B antibody, and an anti-GAPDH antibody.

Accordingly, U87MG cells phase contrast images (Fig. 16) show that PF induced the formation of a high number of vacuoles within the cytoplasm, maybe representative of autophagosomes, and dense bodies scattered throughout the cytoplasm, that could be lysosomes. Notably, as can be appreciated both by AO staining and by phase contrast images (Figs. 14 and 16), most of the U87MG cells exposed to PF showed an irregular shape, being giant and multinucleated, morphologic changes typical of mitotic catastrophe. In particular, multinucleated cells represented about 35% and 73% of total PF-treated cells at 48 and 72 hours of treatment, respectively, while they only account for 4% of total untreated cells at both time considered (Fig. 17).

These findings suggest that in GBM cells mitotic catastrophe could be the mechanism of cell death induced by PF.

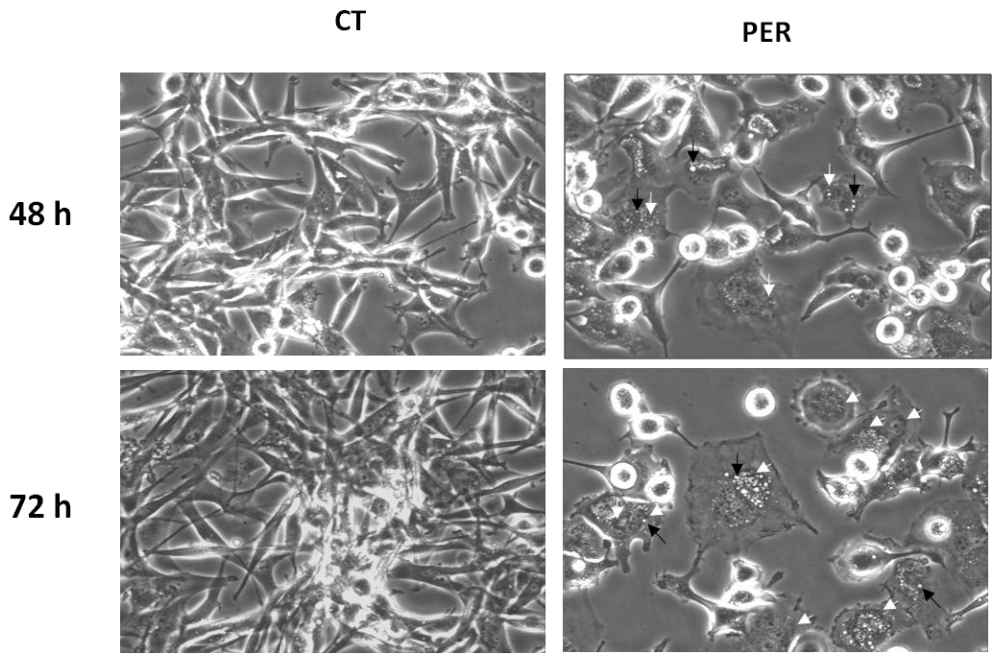


**Figure 16.** *Effect of PF on U87MG cells morphology*. Representative images of U87MG cells morphology after 48 **(A)** and 72 h **(B)** of treatment with or without 10 µM PF. Images were viewed on a contrast phase microscope and digital images were acquired (magnification 20X). Black arrows indicate autophagosomes; white arrows indicate lysosomes (magnification 20X).

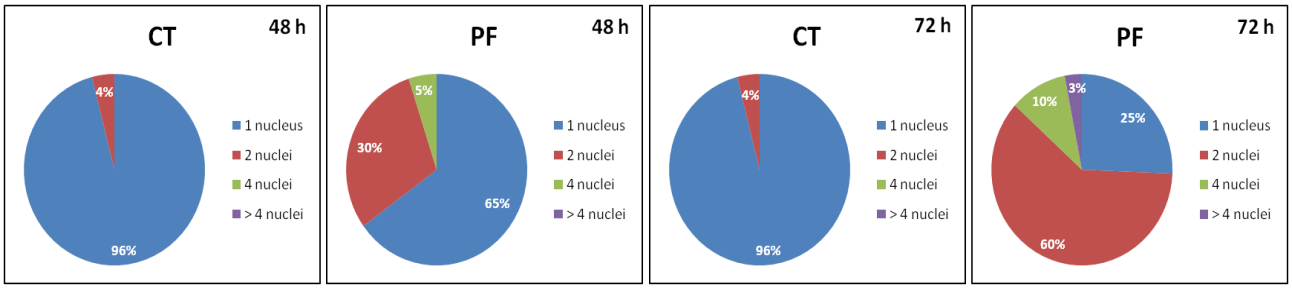


**Figure 17.** *PF induces multinucleation of U87MG cells.* The pie chart shows the percentage of cells with 1, 2, 4 or more than 4 nuclei on total cells of cells treated in absence or presence of 10 µM PF for 48 and 72 h.

## 5.10 Effect of Perifosine on ERK activation

It is known that the Akt/mTOR/p70S6K pathway is the main regulatory pathway that negatively controls autophagy, while the ERK1/2 pathway regulates autophagy positively (Sridharan et al., 2011). Several drugs induce autophagy by inhibiting Akt and, at the same time, activating ERK1/2 (Aoki et al., 2007; Ellington et al., 2006). Since we demonstrated that PF induced autophagy in U87MG cells and, from literature, could inhibit or activate ERK1/2, depending on cell type (Fei et al., 2010; Hideshima et al., 2006; Leleu et al., 2007; Momota et al., 2005; Papa et al., 2008), we analyzed whether PF altered the phosphorylation status of ERK1/2 in U87MG cells through immunoblotting experiments. Our data demonstrated that in our cell model, PF maintains ERK in

its active/phosphorylated state even up to 24 hours of treatment, without altering the total amount of ERK (Fig. 18).

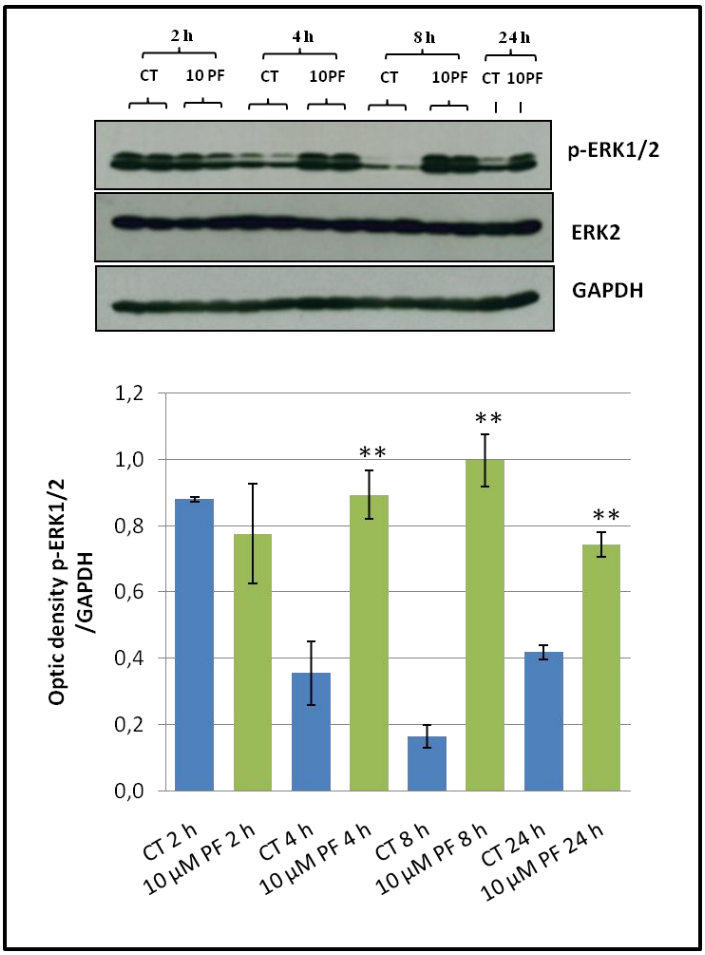


**Figure 18.** *Effect of PF on ERK activation in U87MG cells.* Cells were treated with or without 10 µM PF for 2, 4, 8, and 24 h and then harvested in lysis buffer for immunoblotting analysis of p-ERK, ERK2, and GAPDH levels, as described in "Material and Methods" section. The levels of p-ERK, ERK2, GAPDH were determined by densitometric quantitation and normalized for GAPDH. Values are mean ± SD of three independent experiments. Asterisks represent significant differences between control and treated cells (*p < 0.5; **p < 0.01, Student's *t* test).

## 5.11 Role of ERK1/2 activity in Perifosine-induced autophagy

To determine whether the activity of ERK is involved in PF-induced autophagy, we used a chemical inhibitor of MEK, PD98059 (PD98). As shown in Fig. 19, panel A, pretreatment of U87MG cells with PD98 followed by PF incubation for 4 hours led to a drastic inhibition of ERK1/2 phosphorylation compared with PF-treated cells. Moreover, cells were pretreated or not with PD98 and incubated with PF for 48 hours. Immunoblotting analysis of LC3B conversion from LC3B-I to LC3B-II showed that the pretreatment with PD98 was able to counteract the effect of PF on LC3B-II formation. Indeed, cells treated with PF in combination with PD98 had a significant decreased in

LC3B-II levels than those treated with PF alone (Fig. 19, panel B). These findings demonstrated that PF-induced autophagy requires ERK1/2 activation.

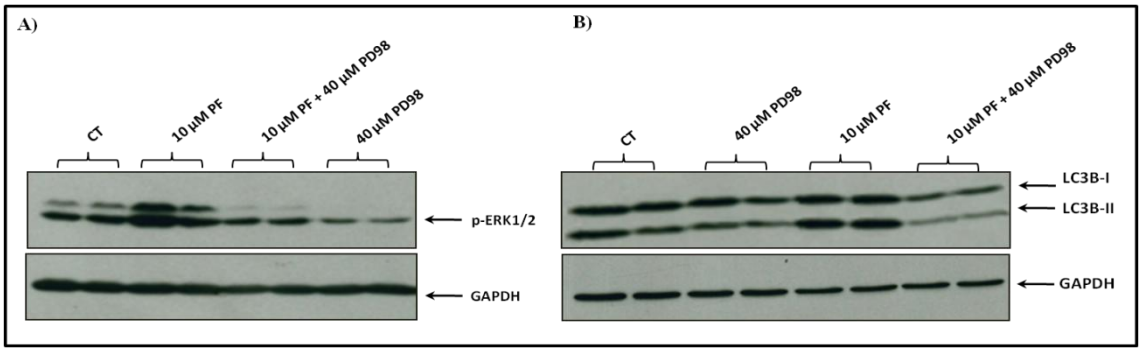


**Figure 19.** (**A)** *Effect of PD98 on ERK activity in U87MG cells treated with PF.* Cells were cultured with control media or pretreated in the absence or presence of 10 μM PD98 1 h prior to treatment with 10 μM PF or 10 μM PD98 for 4 h, as indicated. At the end of treatments, cells were harvested in lysis buffer for immunoblotting analysis, as described in "Material and Methods" section. Equal amounts of protein from each sample were analyzed by immunoblotting with an anti-p-ERK antibody and an anti-GAPDH antibody. **(B)** *Effect of PD98 on LC3B-II amount in U87MG cells treated with PF.* Cells were cultured with control media or pretreated in the absence or presence of 10 μM PD98 1 h prior to treatment with 10 μM PF or 10 μM PD98 for 48 h, as indicated. At the end of treatments, cells were harvested in lysis buffer for immunoblotting analysis, as described in "Material and Methods" section. Equal amounts of protein from each sample were analyzed by immunoblotting with an anti-LC3B antibody, and an anti-GAPDH antibody.

## 5.12 Effect of Perifosine in combination with PD98059 on U87MG cells viability

The results obtained by immunoblotting experiments showed that in U87MG cells PF is able to maintain ERK1/2 in a phosphorylated/active state and that the autophagy induced by PF is also due to ERK1/2 activity. In order to understand the role of ERK1/2 activity in PF-induced cytotoxicity, we evaluated the effect of PF on U87MG cells viability in the absence or presence of PD98 for 48 and 72 hours. As shown in Fig. 20, treatment with PF alone induced a decrease in cell survival of about 25% at 48 hours, in comparison with control cells, while PD98 treatment by itself induced a decrease of cell viability of about 56% at 48 hours, with respect to control. Intriguingly, treatment with PF combined with PD98 further decreased cell survival of about 55% compared to PF alone and of about 22% with respect to cells treated only with PD98. Similar results were obtained at 72 hours of treatment. These findings suggest that ERK1/2 activity protects cells from PF-induced cytotoxicity and, therefore, this molecular mechanism could regulate the resistance to PF, possibly also by inducing autophagy.

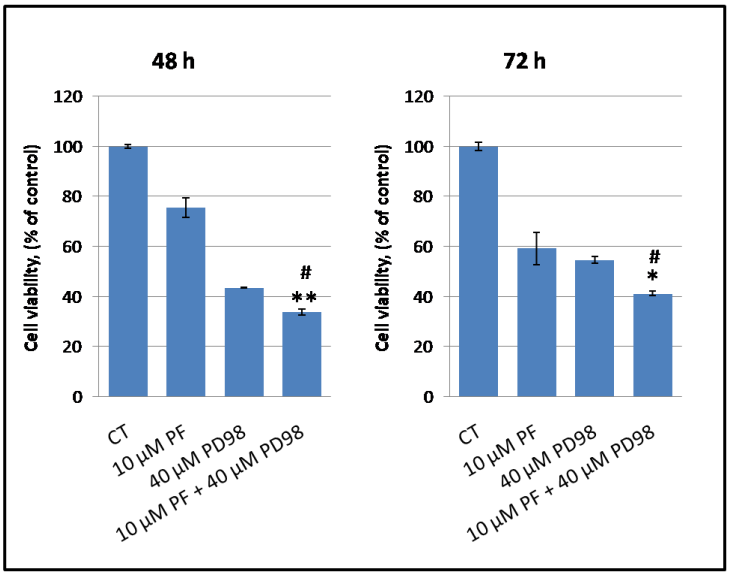


**Figure 20.** *Effect of PF in combination with PD98 on U87MG cells viability.*Cells were cultured with control media or pretreated in the absence or presence of 10 µM PD98 1 h prior to treatment with10 µM PF or 10 µM PD98, as indicated. Cells viability was assessed after 48 and 72 h of treatment by MTT assay. The results are expressed as a percentage of viable cells with respect to control untreated cells and presented as means ± SD of three independent experiments. Asterisks represent statistical significance determined by Student's t test (*$p < 0.05$, **$p < 0.01$ vs PF-treated cells; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs PD98-treated cells).

## 5.13 Effect of Perifosine in combination with D609 on U87MG cells viability

Since we demonstrated that PF is able to reduce the SM level in U87MG cells acting through SMS, we wonder if this effect could be part of the cytotoxic mechanism of PF. To this purpose, we evaluated the effect of a SMS inhibitor, D609, in the absence or presence of PF on U87MG cells viability. As shown in Fig. 21, D609 treatment induced a decrease of cell viability of about 25% and 19% at 48 and 72 hours, respectively, compared to control cells. Moreover, the treatment with PF plus D609 further reduced the viability of cells treated only with PF by about 28% and that of cells treated only with D609 by about 27% at 48 hours. Furthermore, treatment with PF combined with D609 induced a significantly decrease of cell viability by about 18% compared to cells treated only with PF and by about 41% compared to D609-treated cells at 72 hours of treatment. These results indicated that the inhibition of SM synthesis is involved in the cytotoxic effects of PF. However, additional molecular mechanisms are involved in its action.

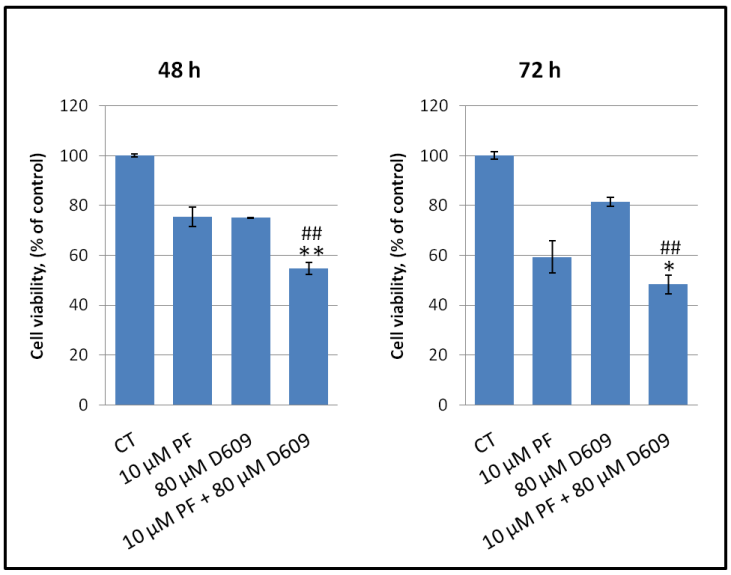


**Figure 21.** *Effect of PF in combination with D609 on U87MG cells viability.* Cells were cultured with control media or pretreated in the absence or presence of 80 µM D609 2 h prior to treatment with10 µM PF or 80 µM D609, as indicated. Cells viability was assessed after 48 and 72 h of treatment by MTT assay. The results are expressed as a percentage of viable cells with respect to control untreated cells and presented as means ± SD of three independent experiments. Asterisks represent statistical significance determined by Student's t test (*$p < 0.05$; **$p < 0.01$ vs PF-treated cells; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs D609-treated cells).

## 5.14 Role of gangliosides in Perifosine-induced toxicity in U87MG cells

The evidence that PF significantly increased the levels of endogenous gangliosides stimulated us to analyze whether the inhibition of gangliosides synthesis might be able to modulate U87MG cells death by preventing the cytotoxic effect of PF.

To inhibit the synthesis of gangliosides and, as a consequence, their total amount in the cells, we inhibited the synthesis of GlcCer, the first GSL in the metabolic pathway for the synthesis of complex GSLs, using two different inhibitors of GCS: PPMP and PDMP. In particular, we evaluated the effect of PF on U87MG cells viability in the absence or presence of PPMP and PDMP, for 48 and 72 hours. As shown in Fig. 22, PPMP and PDMP alone did not induce cytotoxic effects by themselves, but they were able to reduce the cytotoxic effect of PF by inducing an increase in viable cell number of about 28% and 24% respectively at 48 hours of treatment and of about 31% and 42% respectively at 72 hours of treatment compared to cells treated only with PF. Taken together, these results indicate that the co-treatment of PF and PPMP or PDMP rendered U87MG cells resistant to the cytotoxic effect of PF.

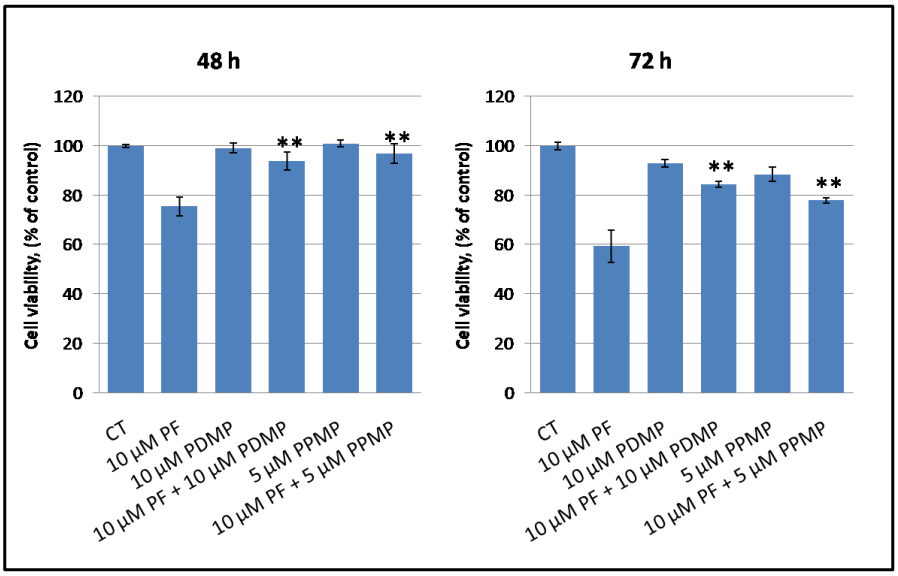


**Figure 22.** *Effect of PF in combination with PPMP or PDMP on U87MG cells viability*. Cells were cultured with control media or pretreated in the absence or presence of 5 µM PPMP or 10 µM PDMP 2 h prior to treatment with 10 µM PF, 5 µM PPMP or 10µM PDMP, as indicated. Cells viability was assessed after 48 and 72 h of treatment by MTT assay. The results are expressed as a percentage of viable cells with respect to control untreated cells and presented as means ± DS of three independent experiments. Asterisks represent statistical significance determined by Student's t test (*$p < 0.05$; **$p < 0.01$ vs PF-treated cells).

# DISCUSSION

GBM is the most frequent and aggressive malignant tumor of the CNS in adults. Despite decades of experimentation to improve the outcome of patients with GBM, this type of neoplasm remains the most lethal one. Therefore, the need to test different and new agents for efficacy and safety is urgent (Rich and Bigner, 2004; Wen and Kesari, 2008). PF, a relatively new compound belonging to the class of APLs, is a promising candidate for cancer chemotherapy, thanks to its rather unique mechanism of action. Indeed, unlike classical anti-cancer drugs, PF targets the cell membranes rather than the DNA (van Blitterswijk and Verheij, 2013). PF exhibits a potent antineoplastic activity against a multitude of cancer cell lines and different tumor models and is currently employed in a great number of phase II clinical trials (Fensterle et al., 2014; Gills and Dennis, 2009). PF can induce apoptosis and/or cell growth arrest in tumor cells, with molecular mechanisms still to be elucidated. Different primary targets have been linked to its anticancer activity, even if inhibition of Akt seems to be the most important one. The survival signaling pathway PI3K/Akt, which is frequently up-regulated in GBM cells, is considered one of the most promising targets for tumor therapy (van Blitterswijk and Verheij, 2013). However, PF can induce also Akt-independent effects and the contribution of Akt inhibition to the clinical activity of PF remains to be assessed (Fensterle et al., 2014; Gills and Dennis, 2009). PF can interfere with the metabolism and transport of membrane lipids and/or the enrichment of PF in cell membranes can directly induce a biophysical disturbance of cell membranes, resulting in alteration of membrane lipid turnover and membrane associated signaling events (including Akt signaling), leading ultimately to cell death (Marco et al., 2014). However, the details of the PF mechanisms of action are still unclear, and this agent has not been studied fully in GBM cells. Our study provides a contribution to this complex field.

First, since PTEN plays a critical role in regulating Akt activity, we examined the effects of PF on cell survival in three human GBM cell lines (T98G, CCF-STTG1 and U87MG), characterized by a different mutation status of PTEN. The PI3K/Akt signaling pathway, which has a crucial role in cell proliferation and survival (Osaki et al., 2004), is frequently upregulated in gliomas through several mechanisms, and is significantly associated with increased tumor grade, decreased levels of apoptosis, and adverse clinical outcome in human gliomas (Chakravarti et al., 2004). Our results demonstrated that PF inhibits cell viability in a dose-dependent manner in all GBM cell lines (Fig. 6). Moreover, comparison of the cell lines suggests that the sensitivity to PF was not correlated to PTEN mutation status and so to basal levels of p-Akt. These findings are consistent with a previous

report showing that PF affects cell viability in lung cancer cells independently of PTEN mutation status (Elrod et al., 2007). Furthermore, we evaluated if PF reduces Akt phosphorylation in our GBM cell lines upon serum stimulation. As expected, we obtained that PF promotes a decrease of p-Akt levels in a time-dependent manner, with a similar trend in all the studied cells (Fig. 7). These results are in agreement with several other studies showing that PF in different tumor cell lines (even in some GBM cell lines) is able to downregulate not only endogenous active Akt, but also serum and growth factor-stimulated Akt (de la Pena et al., 2006; Gills and Dennis, 2009; Zhang et al., 2007a). In parallel, we found that LY29, a potent inhibitor of PI3K (Knight, 2010), induces an almost complete inhibition of Akt after only 1 hour of treatment in the same GBM cell lines (Fig. 7). We consequently wonder if PF is still able to modulate cell viability of U87MG cells exposed to LY29. Our data demonstrated that, even in that condition, namely when Akt is completely inhibited, PF is able to exert further cytotoxic effects, indicating that its action is not solely due to Akt inhibition (Fig. 8).

Accumulating literature indicates that PF could affect cell membrane lipid metabolism and, consequently, cell membranes composition and functionality (Fensterle et al., 2014). In several tumor cell lines it has been shown that PF inhibits the rate limiting step of PC synthesis, which is catalyzed by CT enzyme, inducing cellular stress and subsequently apoptosis in rapidly proliferating cells. However, the mechanism by which PC induces cells death is still unclear. PC is the donor of phosphocholine in the reaction catalyzed by the enzyme SMS (Diringer et al., 1972; Riboni et al., 1997). In accordance with the precursor-product relationship, the inhibition of the PC biosynthesis may also affect the SM biosynthesis from PC and Cer and, as a consequence, may induce an accumulation of the last pro-death lipid. Based on these findings, we examined whether PF is able to inhibit PC and SM biosynthesis in U87MG cells. We found that when using radiolabeled choline as exogenous substrate, exposure of U87MG cells to PF produced a marked inhibition of PC and SM synthesis (Fig. 9). However, even if the PC biosynthesis was reduced, it seems not to be a limiting factor for SM synthesis. Since inhibition of SM biosynthesis should result in increased Cer levels, we investigated the effect of PF on Cer metabolism performing short time pulse experiments in U87MG cells by administering to cells tritiated Sph as Cer precursor. We found that the utilization of newly synthesized Cer was reduced in cell exposed to PF mainly for the biosynthesis of SM and, apparently, also for the biosynthesis of GlcCer and LacCer, without altering the process of Cer synthesis (Fig. 10). Of interest, in a previous study it has been demonstrated that U87MG cells, observed by transmission electron microscopy after exposure to PF or other ALPs, showed many enlarged Golgi cisternae (Rios-Marco et al., 2013). Since the biosynthesis of SM and GlcCer take place at the Golgi level, we could assume that PF could disturb

the Golgi membrane with an impact on SMS and GCS activities. Our results, obtained from SMS and GCS activity assays both in cell culture and cell-free, indicated that PF affects SMS activity without directly interact with the enzyme, while GCS activity is preserved in the same condition (Fig. 11). This discrepancy could be due to the different localization of the enzymes within the Golgi apparatus and the different orientation of their catalytic site, that is luminal for SMS and cytoplasmic for GCS (Schweizer et al., 1994). Based on these findings, we then assessed in U87MG cells the endogenous levels of PLs and gangliosides, as important functional component of cell membranes. We found that PF induced a decrease in the total PLs/protein ratio accompanied by a change in the PLs pattern. Indeed, PF-treated cells were poorer in PC, SM, and PS, and richer in PE than untreated cells (Fig. 12). Surprisingly, PF induces an about 60% increase of endogenous gangliosides levels compared to untreated cells (Fig. 13), indicating that the reduction of GlcCer and LacCer observed in metabolic experiments is not due to the inhibition of their biosynthesis, but rather to their rapid utilization for the synthesis of more complex GSLs. These findings indicate that PF profoundly alters membrane lipid metabolism and content and this could be the main mechanism by which it induces cell death. Analyzing the mechanism of PF-induced cells death in U87MG cells, we found that PF induces autophagy, while apoptosis was undetectable (Figs. 14 and 15). This results was confirmed in the other two GBM cell lines studied, indicating that this could be a general response of GBM cells to PF (Fig. 14). The role of autophagy in cancer therapy is still controversial. It may be the mechanism by which an agent effects cell killing (autophagic cell death or type II programmed cell death) or a tumor cell survival mechanism activated after cancer treatment. When autophagy is protective, its suppression would reduce cell viability, probably by accelerating entry into other forms of cell death (Wu et al., 2012). We demonstrated that, in U87MG cells, PF-induced autophagy requires ERK activity (Figs. 18 and 19). Inhibition of the MAPK pathway suppresses PF-induced autophagy and results in enhanced cytotoxicity (Figs. 19 and 20). In line with our results, previous studies reported that PF induces protective autophagy and confirmed that autophagy prevention is able to significantly enhance PF-induced anti-cancer efficiency (Fu et al., 2009; Qin et al., 2013; Sun, 2010; Tong et al., 2012). In particular, Fu et al. showed that PF induces autophagy in human lung cancer cell lines by inhibiting the Akt/mTOR signaling pathway (Fu et al., 2009). As regard U87MG cell line, conflicting data have been reported showing that PF may induce autophagy (Qin et al., 2013) through the same mechanism reported by Fu et al. or may be inhibitory in the late stages of autophagy, blocking the autophagic flux (Rios-Marco et al., 2015). To the best of our knowledge, we are the first showing that ERK activity is required for PF-induced autophagy. Our data contrast with previous findings showing that ERK was not affected by PF-treatment in U87MG cells (Qin et al., 2013), but agree with those of other

studies in which it has been reported that PF could induce ERK activation in different cells lines (Hideshima et al., 2006; Hou et al., 2014; Leleu et al., 2007). In addition, it has been reported that other drugs, such as curcumin and the natural products triterpenoid B-group soyasaponins induced autophagy by inhibiting Akt signaling and enhancing ERK activity (Aoki et al., 2007; Ellington et al., 2006). The final outcome of autophagy, in terms of survival or death of the cells, would depend on specific cellular setting. In U87MG cells exposed to PF we found a time-dependent increase of cells with an irregular shape and that are giant and multinucleated (Figs. 14, 16, and 17). These morphological changes resemble those described for mitotic catastrophe. Mitotic catastrophe is a mode of cell death that occurs in response to several anticancer agents, which originates from mitotic perturbations detected during mitosis and always accompanied by mitotic arrest (Portugal et al., 2010). Ríos-Marco at al. demonstrated that the exposure of U87MG cells to PF and others APLs induces G2/M phase cell-cycle arrest (Rios-Marco et al., 2013). In particular, ALPs decreased the phosphorylation status of Tyr15 in cdc2 kinase protein, which governs the activity of the cdc2/cyclin B1 complex (responsible for the onset of mitosis), indicating that they provoke mitotic arrest (Rios-Marco et al., 2013). Taken together, these findings indicate that PF in U87MG cells could induce cell death through mitotic catastrophe.

As we reported above, lipid membrane microdomains are important signaling platforms whose structure and functionality are sensitive to membrane lipid composition (Sonnino and Prinetti, 2010). Since we demonstrated that PF induce a pronounced alteration of lipid membrane metabolism and content, these alterations could well affect lipid microdomain functionality and ultimately induce cell death. In particular, we showed that PF affects not only PC biosynthesis, but also glycerophospholipids and SLs ones. SLs are particularly abundant in the plasma membrane, where SM and GSLs represent the major SLs, displaying an asymmetric or polarized distribution, and play important roles in the regulation of membrane fluidity and sub-domain structures (Sonnino and Prinetti, 2010). We demonstrated that SM and GSLs were both affected by PF. Moreover, it has been shown that SM biosynthesis by SMS1 in the Golgi apparatus is crucial for new lipid microdomains formation and for subsequent cycling of these structures towards and from the plasma membrane (Ikonen, 2001; Tafesse et al., 2006; van Blitterswijk et al., 2003). Mimicking the effect of PF on SMS using the D609 compound in U87MG cells, we found a reduction in cell viability increased in combination with PF treatment (Fig. 21). This result indicates that inhibition of SM synthesis is part of the cytotoxic mechanism of PF, but additional molecular mechanisms are involved in its action. One can assume that the cytotoxic effect induced by inhibiting SMS could be also due to the production of Cer at the ER level. However, even if we demonstrated in metabolic studies that PF induces a modest increase in the Cer levels, Qui at al. showed that the endogenous

Cer content is only slightly enhanced after PF exposure (Qin et al., 2013) and, coherently, Ríos-Marco at al. demonstrated that PF and other APLs failed to provoke ER stress in the U87MG cell line (Rios-Marco et al., 2015). These findings indicate that other mechanisms, such as that involved in membrane functionality, are most probably involved in PF-induced cell death. Intriguingly, Ríos-Marco et al. showed that PF and other ALPs induce a deregulation of cholesterol homeostasis that ultimately leads to an accumulation of free cholesterol within the cell and, in particular, in membrane microdomains such as lipid rafts, disrupting their function. However, in U87MG cells they reported an accumulation of free cholesterol in the cytoplasmic compartment, but not at the plasma membrane level (Rios-Marco et al., 2013). Herein, we showed that PF enormously enhances gangliosides endogenous content (Fig. 13). Gangliosides reside mostly in the outer leaflet of the plasma membranes, where they cluster to forms GSLs-enriched microdomains, which provide a microenvironment for reciprocal interactions between lipids and protein molecules involved in the control of signal transduction (Sonnino and Prinetti, 2010). GSLs show a specific distribution on the membrane for each cell type, which can vary with differentiation and neoplastic transformation (Huwiler et al., 2000; Kaucic et al., 2001; Segui et al., 2006). In U87MG cells, we found that inhibiting the first step in the metabolic pathway of the synthesis of complex GSLs, including gangliosides, by the use of two different GCS inhibitors (PPMP and PDMP), was paralleled by an increase in cell resistance to the cytotoxic effect of PF (Fig. 22). Recently, an interested study reported that gangliosides may play a role in the localization of the APL Edelfosine to lipid rafts, due to its stronger affinity for gangliosides than for SM (Hac-Wydro and Dynarowicz-Latka, 2010). Since Edelfosine is structurally related to PF, this could rise the hypothesis that also PF, as well as other APLs, have a high affinity to ganglosides. Consequently, gangliosides could have a role also in APLs uptake. To the best of our knowledge, we are the first showing that gangliosides are major player in mediating the cytotoxic action of PF.

In conclusion, PF treatment in GBM cells results in a complex network of effects where the alteration of the metabolism and content of membrane-lipid components (and maybe their related secondary messengers) seems to play a key role in determining cell death. In this scenario, gangliosides are the main players. This study indicates that PF is decisive in its target to fight GBM cells, this representing a critical push to study new aspects in its cellular mechanisms, implying PF as anti-GBM agent.

# REFERENCE


Adan-Gokbulut, A., Kartal-Yandim, M., Iskender, G., and Baran, Y. (2013). Novel agents targeting bioactive sphingolipids for the treatment of cancer. Current medicinal chemistry *20*, 108-122.

Alderliesten, M.C., Klarenbeek, J.B., van der Luit, A.H., van Lummel, M., Jones, D.R., Zerp, S., Divecha, N., Verheij, M., and van Blitterswijk, W.J. (2011). Phosphoinositide phosphatase SHIP-1 regulates apoptosis induced by edelfosine, Fas ligation and DNA damage in mouse lymphoma cells. The Biochemical journal *440*, 127-135.

Angstrom, J., Teneberg, S., and Karlsson, K.A. (1994). Delineation and comparison of ganglioside-binding epitopes for the toxins of Vibrio cholerae, Escherichia coli, and Clostridium tetani: evidence for overlapping epitopes. Proceedings of the National Academy of Sciences of the United States of America *91*, 11859-11863.

Aoki, H., Takada, Y., Kondo, S., Sawaya, R., Aggarwal, B.B., and Kondo, Y. (2007). Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Molecular pharmacology *72*, 29-39.

Arthur, G., and Bittman, R. (2014). Glycosylated antitumor ether lipids: activity and mechanism of action. Anti-cancer agents in medicinal chemistry *14*, 592-606.

Asthagiri, A.R., Pouratian, N., Sherman, J., Ahmed, G., and Shaffrey, M.E. (2007). Advances in brain tumor surgery. Neurologic clinics *25*, 975-1003, viii-ix.

Bach, D., Sela, B., and Miller, I.R. (1982). Compositional aspects of lipid hydration. Chemistry and physics of lipids *31*, 381-394.

Bakht, O., Pathak, P., and London, E. (2007). Effect of the structure of lipids favoring disordered domain formation on the stability of cholesterol-containing ordered domains (lipid rafts): identification of multiple raft-stabilization mechanisms. Biophysical journal *93*, 4307-4318.

Bartlett, G.R. (1959). Colorimetric assay methods for free and phosphorylated glyceric acids. The Journal of biological chemistry *234*, 469-471.

Becher, O.J., Hambardzumyan, D., Walker, T.R., Helmy, K., Nazarian, J., Albrecht, S., Hiner, R.L., Gall, S., Huse, J.T., Jabado, N.*, et al.* (2010). Preclinical evaluation of radiation and perifosine in a genetically and histologically accurate model of brainstem glioma. Cancer research *70*, 2548-2557.

Behin, A., Hoang-Xuan, K., Carpentier, A.F., and Delattre, J.Y. (2003). Primary brain tumours in adults. Lancet *361*, 323-331.

Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998). Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene *17*, 313-325.

Berdel, W.E., Bausert, W.R., Weltzien, H.U., Modolell, M.L., Widmann, K.H., and Munder, P.G. (1980). The influence of alkyl-lysophospholipids and lysophospholipid-activated macrophages on the development of metastasis of 3-Lewis lung carcinoma. European journal of cancer *16*, 1199-1204.

Berger, M.R., Munder, P.G., Schmahl, D., and Westphal, O. (1984). Influence of the alkyllysophospholipid ET-18-OCH3 on methylnitrosourea-induced rat mammary carcinomas. Oncology *41*, 109-113.

Brocca, P., Berthault, P., and Sonnino, S. (1998). Conformation of the oligosaccharide chain of G(M1) ganglioside in a carbohydrate-enriched surface. Biophysical journal *74*, 309-318.

Brown, D.A. (2006). Lipid rafts, detergent-resistant membranes, and raft targeting signals. Physiology (Bethesda) *21*, 430-439.

Buckner, J.C., Brown, P.D., O'Neill, B.P., Meyer, F.B., Wetmore, C.J., and Uhm, J.H. (2007). Central nervous system tumors. Mayo Clinic proceedings *82*, 1271-1286.

Cantu, L., Corti, M., Sonnino, S., and Tettamanti, G. (1990). Evidence for spontaneous segregation phenomena in mixed micelles of gangliosides. Chemistry and physics of lipids *55*, 223-229.

Carrasco, M.P., Jimenez-Lopez, J.M., Rios-Marco, P., Segovia, J.L., and Marco, C. (2010). Disruption of cellular cholesterol transport and homeostasis as a novel mechanism of action of membrane-targeted alkylphospholipid analogues. British journal of pharmacology *160*, 355-366.

Castro, B.M., Fedorov, A., Hornillos, V., Delgado, J., Acuna, A.U., Mollinedo, F., and Prieto, M. (2013). Edelfosine and miltefosine effects on lipid raft properties: membrane biophysics in cell death by antitumor lipids. The journal of physical chemistry. B *117*, 7929-7940.

Chakravarti, A., Zhai, G., Suzuki, Y., Sarkesh, S., Black, P.M., Muzikansky, A., and Loeffler, J.S. (2004). The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *22*, 1926-1933.

Charles, N.A., Holland, E.C., Gilbertson, R., Glass, R., and Kettenmann, H. (2011). The brain tumor microenvironment. Glia *59*, 1169-1180.

Chazotte, B. (2012). Labeling Golgi with fluorescent ceramides. Cold Spring Harbor protocols *2012*.

Chen, M.B., Wu, X.Y., Tao, G.Q., Liu, C.Y., Chen, J., Wang, L.Q., and Lu, P.H. (2012). Perifosine sensitizes curcumin-induced anti-colorectal cancer effects by targeting multiple signaling pathways both in vivo and in vitro. International journal of cancer. Journal international du cancer *131*, 2487-2498.

Chiantia, S., and London, E. (2013). Sphingolipids and membrane domains: recent advances. Handbook of experimental pharmacology, 33-55.

Chiarini, F., Del Sole, M., Mongiorgi, S., Gaboardi, G.C., Cappellini, A., Mantovani, I., Follo, M.Y., McCubrey, J.A., and Martelli, A.M. (2008). The novel Akt inhibitor, perifosine, induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug-resistant human T-acute leukemia cells by a JNK-dependent mechanism. Leukemia *22*, 1106-1116.

Clarke, C.J., Snook, C.F., Tani, M., Matmati, N., Marchesini, N., and Hannun, Y.A. (2006). The extended family of neutral sphingomyelinases. Biochemistry *45*, 11247-11256.

Clarke, J., Butowski, N., and Chang, S. (2010). Recent advances in therapy for glioblastoma. Archives of neurology *67*, 279-283.

Coskun, U., and Simons, K. (2010). Membrane rafting: from apical sorting to phase segregation. FEBS letters *584*, 1685-1693.

de la Pena, L., Burgan, W.E., Carter, D.J., Hollingshead, M.G., Satyamitra, M., Camphausen, K., and Tofilon, P.J. (2006). Inhibition of Akt by the alkylphospholipid perifosine does not enhance the radiosensitivity of human glioma cells. Molecular cancer therapeutics *5*, 1504-1510.

de Vries, N.A., Beijnen, J.H., Boogerd, W., and van Tellingen, O. (2006). Blood-brain barrier and chemotherapeutic treatment of brain tumors. Expert review of neurotherapeutics *6*, 1199-1209.

Degroote, S., Wolthoorn, J., and van Meer, G. (2004). The cell biology of glycosphingolipids. Seminars in cell & developmental biology *15*, 375-387.

Dick, J.E. (2008). Stem cell concepts renew cancer research. Blood *112*, 4793-4807.

Dineva, I.K., Zaharieva, M.M., Konstantinov, S.M., Eibl, H., and Berger, M.R. (2012). Erufosine suppresses breast cancer in vitro and in vivo for its activity on PI3K, c-Raf and Akt proteins. Journal of cancer research and clinical oncology *138*, 1909-1917.

Diringer, H., Marggraf, W.D., Koch, M.A., and Anderer, F.A. (1972). Evidence for a new biosynthetic pathway of sphingomyelin in SV 40 transformed mouse cells. Biochemical and biophysical research communications *47*, 1345-1352.

Dolecek, T.A., Propp, J.M., Stroup, N.E., and Kruchko, C. (2012). CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. Neuro-oncology *14 Suppl 5*, v1-49.

Duan, R.D. (2006). Alkaline sphingomyelinase: an old enzyme with novel implications. Biochimica et biophysica acta *1761*, 281-291.

Dumontet, C., Thomas, L., Berard, F., Gimonet, J.F., and Coiffier, B. (2006). A phase II trial of miltefosine in patients with cutaneous T-cell lymphoma. Bulletin du cancer *93*, E115-118.

Dymond, M., Attard, G., and Postle, A.D. (2008). Testing the hypothesis that amphiphilic antineoplastic lipid analogues act through reduction of membrane curvature elastic stress. Journal of the Royal Society, Interface / the Royal Society *5*, 1371-1386.

Eibl, H., Arnold, D., Weltzien, H.U., and Westphal, O. (1967). [On the synthesis of alpha and beta lecithins and their ether analogs]. Justus Liebigs Annalen der Chemie *709*, 226-230.

Eibl, H., and Unger, C. (1990). Hexadecylphosphocholine: a new and selective antitumor drug. Cancer treatment reviews *17*, 233-242.

Ellington, A.A., Berhow, M.A., and Singletary, K.W. (2006). Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells. Carcinogenesis *27*, 298-306.

Elrod, H.A., Lin, Y.D., Yue, P., Wang, X., Lonial, S., Khuri, F.R., and Sun, S.Y. (2007). The alkylphospholipid perifosine induces apoptosis of human lung cancer cells requiring inhibition of Akt and activation of the extrinsic apoptotic pathway. Molecular cancer therapeutics *6*, 2029-2038.

Erdlenbruch, B., Jendrossek, V., Gerriets, A., Vetterlein, F., Eibl, H., and Lakomek, M. (1999). Erucylphosphocholine: pharmacokinetics, biodistribution and CNS-accumulation in the rat after intravenous administration. Cancer chemotherapy and pharmacology *44*, 484-490.

Erdlenbruch, B., Jendrossek, V., Marx, M., Hunold, A., Eibl, H., and Lakomek, M. (1998). Antitumor effects of erucylphosphocholine on brain tumor cells in vitro and in vivo. Anticancer research *18*, 2551-2557.

Fei, H.R., Chen, G., Wang, J.M., and Wang, F.Z. (2010). Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation. Cytotechnology *62*, 449-460.

Feizi, T. (1985). Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. Nature *314*, 53-57.

Fensterle, J., Aicher, B., Seipelt, I., Teifel, M., and Engel, J. (2014). Current view on the mechanism of action of perifosine in cancer. Anti-cancer agents in medicinal chemistry *14*, 629-635.

Fine, H.A., Dear, K.B., Loeffler, J.S., Black, P.M., and Canellos, G.P. (1993). Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. Cancer *71*, 2585-2597.

Floryk, D., and Thompson, T.C. (2008). Perifosine induces differentiation and cell death in prostate cancer cells. Cancer letters *266*, 216-226.

Fu, L., Kim, Y.A., Wang, X., Wu, X., Yue, P., Lonial, S., Khuri, F.R., and Sun, S.Y. (2009). Perifosine inhibits mammalian target of rapamycin signaling through facilitating degradation of major components in the mTOR axis and induces autophagy. Cancer research *69*, 8967-8976.

Furnari, F.B., Fenton, T., Bachoo, R.M., Mukasa, A., Stommel, J.M., Stegh, A., Hahn, W.C., Ligon, K.L., Louis, D.N., Brennan, C.*, et al.* (2007). Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes & development *21*, 2683-2710.

Futerman, A.H., and Hannun, Y.A. (2004). The complex life of simple sphingolipids. EMBO reports *5*, 777-782.

Gajate, C., and Mollinedo, F. (2014). Lipid rafts, endoplasmic reticulum and mitochondria in the antitumor action of the alkylphospholipid analog edelfosine. Anti-cancer agents in medicinal chemistry *14*, 509-527.

Gajate, C., Santos-Beneit, A., Modolell, M., and Mollinedo, F. (1998). Involvement of c-Jun NH2-terminal kinase activation and c-Jun in the induction of apoptosis by the ether phospholipid 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine. Molecular pharmacology *53*, 602-612.

Gault, C.R., Obeid, L.M., and Hannun, Y.A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. Advances in experimental medicine and biology *688*, 1-23.

Giantonio, B.J., Derry, C., McAleer, C., McPhillips, J.J., and O'Dwyer, P.J. (2004). Phase I and pharmacokinetic study of the cytotoxic ether lipid ilmofosine administered by weekly two-hour infusion in patients with advanced solid tumors. Clinical cancer research : an official journal of the American Association for Cancer Research *10*, 1282-1288.

Gills, J.J., and Dennis, P.A. (2009). Perifosine: update on a novel Akt inhibitor. Current oncology reports *11*, 102-110.

Giussani, P., Maceyka, M., Le Stunff, H., Mikami, A., Lepine, S., Wang, E., Kelly, S., Merrill, A.H., Jr., Milstien, S., and Spiegel, S. (2006). Sphingosine-1-phosphate phosphohydrolase regulates endoplasmic reticulum-to-golgi trafficking of ceramide. Molecular and cellular biology *26*, 5055-5069.

Giussani, P., Tringali, C., Riboni, L., Viani, P., and Venerando, B. (2014). Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. International journal of molecular sciences *15*, 4356-4392.

Goni, F.M., and Alonso, A. (2006). Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. Biochimica et biophysica acta *1758*, 1902-1921.

Goni, F.M., and Alonso, A. (2009). Effects of ceramide and other simple sphingolipids on membrane lateral structure. Biochimica et biophysica acta *1788*, 169-177.

Grassme, H., Cremesti, A., Kolesnick, R., and Gulbins, E. (2003). Ceramide-mediated clustering is required for CD95-DISC formation. Oncogene *22*, 5457-5470.

Ha, J.H., Spolar, R.S., and Record, M.T., Jr. (1989). Role of the hydrophobic effect in stability of site-specific protein-DNA complexes. Journal of molecular biology *209*, 801-816.

Hac-Wydro, K., and Dynarowicz-Latka, P. (2010). Searching for the role of membrane sphingolipids in selectivity of antitumor ether lipid-edelfosine. Colloids and surfaces. B, Biointerfaces *81*, 492-497.

Hakomori, S. (1990). Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. The Journal of biological chemistry *265*, 18713-18716.

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nature reviews. Molecular cell biology *9*, 139-150.

Hayakawa, Y., Godfrey, D.I., and Smyth, M.J. (2004). Alpha-galactosylceramide: potential immunomodulatory activity and future application. Current medicinal chemistry *11*, 241-252.

Heatley, F., and Scott, J.E. (1988). A water molecule participates in the secondary structure of hyaluronan. The Biochemical journal *254*, 489-493.

Henke, G., Lindner, L.H., Vogeser, M., Eibl, H.J., Worner, J., Muller, A.C., Bamberg, M., Wachholz, K., Belka, C., and Jendrossek, V. (2009). Pharmacokinetics and biodistribution of Erufosine in nude mice--implications for combination with radiotherapy. Radiat Oncol *4*, 46.

Hennessy, B.T., Lu, Y., Poradosu, E., Yu, Q., Yu, S., Hall, H., Carey, M.S., Ravoori, M., Gonzalez-Angulo, A.M., Birch, R.*, et al.* (2007). Pharmacodynamic markers of perifosine efficacy. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 7421-7431.

Herrmann, D.B., Pahlke, W., Opitz, H.G., and Bicker, U. (1990). In vivo antitumor activity of ilmofosine. Cancer treatment reviews *17*, 247-252.

Hideshima, T., Catley, L., Yasui, H., Ishitsuka, K., Raje, N., Mitsiades, C., Podar, K., Munshi, N.C., Chauhan, D., Richardson, P.G.*, et al.* (2006). Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood *107*, 4053-4062.

Hilgard, P., Klenner, T., Stekar, J., Nossner, G., Kutscher, B., and Engel, J. (1997). D-21266, a new heterocyclic alkylphospholipid with antitumour activity. European journal of cancer *33*, 442-446.

Holand, K., Salm, F., and Arcaro, A. (2011). The phosphoinositide 3-kinase signaling pathway as a therapeutic target in grade IV brain tumors. Current cancer drug targets *11*, 894-918.

Holland, E.C. (2000). Glioblastoma multiforme: the terminator. Proceedings of the National Academy of Sciences of the United States of America *97*, 6242-6244.

Hou, X., Chen, X., Zhang, P., Fan, Y., Ma, A., Pang, T., Song, Z., Jin, Y., Hao, W., Liu, F.*, et al.* (2014). Inhibition of hedgehog signaling by GANT58 induces apoptosis and shows synergistic antitumor activity with AKT inhibitor in acute T cell leukemia cells. Biochimie *101*, 50-59.

Huwiler, A., Kolter, T., Pfeilschifter, J., and Sandhoff, K. (2000). Physiology and pathophysiology of sphingolipid metabolism and signaling. Biochimica et biophysica acta *1485*, 63-99.

Ichikawa, S., and Hirabayashi, Y. (1998). Glucosylceramide synthase and glycosphingolipid synthesis. Trends in cell biology *8*, 198-202.

Ichimura, K., Ohgaki, H., Kleihues, P., and Collins, V.P. (2004). Molecular pathogenesis of astrocytic tumours. Journal of neuro-oncology *70*, 137-160.

Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahangeer, S., and Nakamura, S. (2003). Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. The Journal of biological chemistry *278*, 46832-46839.

Ikonen, E. (2001). Roles of lipid rafts in membrane transport. Current opinion in cell biology *13*, 470-477.

Jain, R.K., di Tomaso, E., Duda, D.G., Loeffler, J.S., Sorensen, A.G., and Batchelor, T.T. (2007). Angiogenesis in brain tumours. Nature reviews. Neuroscience *8*, 610-622.

Jeckel, D., Karrenbauer, A., Burger, K.N., van Meer, G., and Wieland, F. (1992). Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. The Journal of cell biology *117*, 259-267.

Ji, C., Yang, Y.L., Yang, Z., Tu, Y., Cheng, L., Chen, B., Xia, J.P., Sun, W.L., Su, Z.L., He, L.*, et al.* (2012). Perifosine sensitizes UVB-induced apoptosis in skin cells: new implication of skin cancer prevention? Cellular signalling *24*, 1781-1789.

Jimenez-Lopez, J.M., Rios-Marco, P., Marco, C., Segovia, J.L., and Carrasco, M.P. (2010). Alterations in the homeostasis of phospholipids and cholesterol by antitumor alkylphospholipids. Lipids in health and disease *9*, 33.

Kaucic, K., Etue, N., LaFleur, B., Woods, W., and Ladisch, S. (2001). Neuroblastomas of infancy exhibit a characteristic ganglioside pattern. Cancer *91*, 785-793.

Keusch, G.T., Jacewicz, M., Mobassaleh, M., and Donohue-Rolfe, A. (1991). Shiga toxin: intestinal cell receptors and pathophysiology of enterotoxic effects. Reviews of infectious diseases *13 Suppl 4*, S304-310.

Knight, Z.A. (2010). Small molecule inhibitors of the PI3-kinase family. Current topics in microbiology and immunology *347*, 263-278.

Kondapaka, S.B., Singh, S.S., Dasmahapatra, G.P., Sausville, E.A., and Roy, K.K. (2003). Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Molecular cancer therapeutics *2*, 1093-1103.

Korkaya, H., Paulson, A., Charafe-Jauffret, E., Ginestier, C., Brown, M., Dutcher, J., Clouthier, S.G., and Wicha, M.S. (2009). Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. PLoS biology *7*, e1000121.

Kotting, J., Marschner, N.W., Neumuller, W., Unger, C., and Eibl, H. (1992). Hexadecylphosphocholine and octadecyl-methyl-glycero-3-phosphocholine: a comparison of hemolytic activity, serum binding and tissue distribution. Progress in experimental tumor research *34*, 131-142.

Kuerschner, L., Richter, D., Hannibal-Bach, H.K., Gaebler, A., Shevchenko, A., Ejsing, C.S., and Thiele, C. (2012). Exogenous ether lipids predominantly target mitochondria. PloS one *7*, e31342.

Lahiri, S., and Futerman, A.H. (2007). The metabolism and function of sphingolipids and glycosphingolipids. Cellular and molecular life sciences : CMLS *64*, 2270-2284.

Laquintana, V., Trapani, A., Denora, N., Wang, F., Gallo, J.M., and Trapani, G. (2009). New strategies to deliver anticancer drugs to brain tumors. Expert opinion on drug delivery *6*, 1017-1032.

Le Stunff, H., Giussani, P., Maceyka, M., Lepine, S., Milstien, S., and Spiegel, S. (2007). Recycling of sphingosine is regulated by the concerted actions of sphingosine-1-phosphate phosphohydrolase 1 and sphingosine kinase 2. The Journal of biological chemistry *282*, 34372-34380.

Le Stunff, H., Peterson, C., Thornton, R., Milstien, S., Mandala, S.M., and Spiegel, S. (2002). Characterization of murine sphingosine-1-phosphate phosphohydrolase. The Journal of biological chemistry *277*, 8920-8927.

Leleu, X., Jia, X., Runnels, J., Ngo, H.T., Moreau, A.S., Farag, M., Spencer, J.A., Pitsillides, C.M., Hatjiharissi, E., Roccaro, A.*, et al.* (2007). The Akt pathway regulates survival and homing in Waldenstrom macroglobulinemia. Blood *110*, 4417-4426.

Leonard, R., Hardy, J., van Tienhoven, G., Houston, S., Simmonds, P., David, M., and Mansi, J. (2001). Randomized, double-blind, placebo-controlled, multicenter trial of 6% miltefosine solution, a topical chemotherapy in cutaneous metastases from breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *19*, 4150-4159.

Levy, M., and Futerman, A.H. (2010). Mammalian ceramide synthases. IUBMB life *62*, 347-356.

Li, X., Luwor, R., Lu, Y., Liang, K., and Fan, Z. (2006). Enhancement of antitumor activity of the anti-EGF receptor monoclonal antibody cetuximab/C225 by perifosine in PTEN-deficient cancer cells. Oncogene *25*, 525-535.

Lingwood, D., and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. Science *327*, 46-50.

Lo, H.W. (2010). Targeting Ras-RAF-ERK and its interactive pathways as a novel therapy for malignant gliomas. Current cancer drug targets *10*, 840-848.

LoPiccolo, J., Blumenthal, G.M., Bernstein, W.B., and Dennis, P.A. (2008). Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy *11*, 32-50.

Louis, D.N., Pomeroy, S.L., and Cairncross, J.G. (2002). Focus on central nervous system neoplasia. Cancer cell *1*, 125-128.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of biological chemistry *193*, 265-275.

Maher, E.A., Furnari, F.B., Bachoo, R.M., Rowitch, D.H., Louis, D.N., Cavenee, W.K., and DePinho, R.A. (2001). Malignant glioma: genetics and biology of a grave matter. Genes & development *15*, 1311-1333.

Mao, C., and Obeid, L.M. (2008). Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate. Biochimica et biophysica acta *1781*, 424-434.

Marchesini, N., and Hannun, Y.A. (2004). Acid and neutral sphingomyelinases: roles and mechanisms of regulation. Biochemistry and cell biology = Biochimie et biologie cellulaire *82*, 27-44.

Marco, C., Jimenez-Lopez, J.M., Rios-Marco, P., Segovia, J.L., and Carrasco, M.P. (2009). Hexadecylphosphocholine alters nonvesicular cholesterol traffic from the plasma membrane to the endoplasmic reticulum and inhibits the synthesis of sphingomyelin in HepG2 cells. The international journal of biochemistry & cell biology *41*, 1296-1303.

Marco, C., Rios-Marco, P., Jimenez-Lopez, J.M., Segovia, J.L., and Carrasco, M.P. (2014). Antitumoral alkylphospholipids alter cell lipid metabolism. Anti-cancer agents in medicinal chemistry *14*, 545-558.

Mendez-Otero, R., and Cavalcante, L.A. (2003). Functional role of gangliosides in neuronal motility. Progress in molecular and subcellular biology *32*, 97-124.

Merlo, A. (2003). Genes and pathways driving glioblastomas in humans and murine disease models. Neurosurgical review *26*, 145-158.

Michel, C., and van Echten-Deckert, G. (1997). Conversion of dihydroceramide to ceramide occurs at the cytosolic face of the endoplasmic reticulum. FEBS letters *416*, 153-155.

Miller, C.R., and Perry, A. (2007). Glioblastoma. Archives of pathology & laboratory medicine *131*, 397-406.

Mizushima, N., and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. Autophagy *3*, 542-545.

Mollinedo, F. (2014). Editorial: Antitumor alkylphospholipid analogs: a promising and growing family of synthetic cell membrane-targeting molecules for cancer treatment). Anti-cancer agents in medicinal chemistry *14*, 495-498.

Mollinedo, F., de la Iglesia-Vicente, J., Gajate, C., Estella-Hermoso de Mendoza, A., Villa-Pulgarin, J.A., de Frias, M., Roue, G., Gil, J., Colomer, D., Campanero, M.A*., et al.* (2010). In vitro and In vivo selective antitumor activity of Edelfosine against mantle cell lymphoma and chronic lymphocytic leukemia involving lipid rafts. Clinical cancer research : an official journal of the American Association for Cancer Research *16*, 2046-2054.

Mollinedo, F., Fernandez-Luna, J.L., Gajate, C., Martin-Martin, B., Benito, A., Martinez-Dalmau, R., and Modolell, M. (1997). Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH3 (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X(L). Cancer research *57*, 1320-1328.

Mollinedo, F., Fernandez, M., Hornillos, V., Delgado, J., Amat-Guerri, F., Acuna, A.U., Nieto-Miguel, T., Villa-Pulgarin, J.A., Gonzalez-Garcia, C., Cena, V*., et al.* (2011). Involvement of lipid rafts in the localization and dysfunction effect of the antitumor ether phospholipid edelfosine in mitochondria. Cell death & disease *2*, e158.

Mollinedo, F., and Gajate, C. (2015). Lipid rafts as major platforms for signaling regulation in cancer. Advances in biological regulation *57*, 130-146.

Momota, H., Nerio, E., and Holland, E.C. (2005). Perifosine inhibits multiple signaling pathways in glial progenitors and cooperates with temozolomide to arrest cell proliferation in gliomas in vivo. Cancer research *65*, 7429-7435.

Morad, S.A., and Cabot, M.C. (2013). Ceramide-orchestrated signalling in cancer cells. Nature reviews. Cancer *13*, 51-65.

Mrugala, M.M. (2013). Advances and challenges in the treatment of glioblastoma: a clinician's perspective. Discovery medicine *15*, 221-230.

Munoz-Martinez, F., Torres, C., Castanys, S., and Gamarro, F. (2008). The anti-tumor alkylphospholipid perifosine is internalized by an ATP-dependent translocase activity across the plasma membrane of human KB carcinoma cells. Biochimica et biophysica acta *1778*, 530-540.

Munoz-Martinez, F., Torres, C., Castanys, S., and Gamarro, F. (2010). CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells. Biochemical pharmacology *80*, 793-800.

Neuwelt, E.A., Bauer, B., Fahlke, C., Fricker, G., Iadecola, C., Janigro, D., Leybaert, L., Molnar, Z., O'Donnell, M.E., Povlishock, J.T.*, et al.* (2011). Engaging neuroscience to advance translational research in brain barrier biology. Nature reviews. Neuroscience *12*, 169-182.

Osaki, M., Oshimura, M., and Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis : an international journal on programmed cell death *9*, 667-676.

Palestini, P., Allietta, M., Sonnino, S., Tettamanti, G., Thompson, T.E., and Tillack, T.W. (1995). Gel phase preference of ganglioside GM1 at low concentration in two-component, two-phase phosphatidylcholine bilayers depends upon the ceramide moiety. Biochimica et biophysica acta *1235*, 221-230.

Papa, V., Tazzari, P.L., Chiarini, F., Cappellini, A., Ricci, F., Billi, A.M., Evangelisti, C., Ottaviani, E., Martinelli, G., Testoni, N.*, et al.* (2008). Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor perifosine in acute myelogenous leukemia cells. Leukemia *22*, 147-160.

Pascher, I. (1976). Molecular arrangements in sphingolipids. Conformation and hydrogen bonding of ceramide and their implication on membrane stability and permeability. Biochimica et biophysica acta *455*, 433-451.

Patel, V., Lahusen, T., Sy, T., Sausville, E.A., Gutkind, J.S., and Senderowicz, A.M. (2002). Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. Cancer research *62*, 1401-1409.

Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A.H. (2006). When do Lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis. The Journal of biological chemistry *281*, 25001-25005.

Pike, L.J. (2009). The challenge of lipid rafts. Journal of lipid research *50 Suppl*, S323-328.

Pinton, G., Manente, A.G., Angeli, G., Mutti, L., and Moro, L. (2012). Perifosine as a potential novel anti-cancer agent inhibits EGFR/MET-AKT axis in malignant pleural mesothelioma. PloS one *7*, e36856.

Pitter, K.L., Galban, C.J., Galban, S., Tehrani, O.S., Li, F., Charles, N., Bradbury, M.S., Becher, O.J., Chenevert, T.L., Rehemtulla, A.*, et al.* (2011). Perifosine and CCI 779 co-operate to induce cell death and decrease proliferation in PTEN-intact and PTEN-deficient PDGF-driven murine glioblastoma. PloS one *6*, e14545.

Porta, C., and Figlin, R.A. (2009). Phosphatidylinositol-3-kinase/Akt signaling pathway and kidney cancer, and the therapeutic potential of phosphatidylinositol-3-kinase/Akt inhibitors. The Journal of urology *182*, 2569-2577.

Portugal, J., Mansilla, S., and Bataller, M. (2010). Mechanisms of drug-induced mitotic catastrophe in cancer cells. Current pharmaceutical design *16*, 69-78.

Prinetti, A., Loberto, N., Chigorno, V., and Sonnino, S. (2009). Glycosphingolipid behaviour in complex membranes. Biochimica et biophysica acta *1788*, 184-193.

Pyne, S., Lee, S.C., Long, J., and Pyne, N.J. (2009). Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate signalling in health and disease. Cellular signalling *21*, 14-21.

Qin, L.S., Yu, Z.Q., Zhang, S.M., Sun, G., Zhu, J., Xu, J., Guo, J., and Fu, L.S. (2013). The short chain cell-permeable ceramide (C6) restores cell apoptosis and perifosine sensitivity in cultured glioblastoma cells. Molecular biology reports *40*, 5645-5655.

Rahmani, M., Reese, E., Dai, Y., Bauer, C., Payne, S.G., Dent, P., Spiegel, S., and Grant, S. (2005). Coadministration of histone deacetylase inhibitors and perifosine synergistically induces apoptosis in human leukemia cells through Akt and ERK1/2 inactivation and the generation of ceramide and reactive oxygen species. Cancer research *65*, 2422-2432.

Riboni, L., Giussani, P., and Viani, P. (2010). Sphingolipid transport. Advances in experimental medicine and biology *688*, 24-45.

Riboni, L., Viani, P., Bassi, R., Prinetti, A., and Tettamanti, G. (1997). The role of sphingolipids in the process of signal transduction. Progress in lipid research *36*, 153-195.

Rich, J.N., and Bigner, D.D. (2004). Development of novel targeted therapies in the treatment of malignant glioma. Nature reviews. Drug discovery *3*, 430-446.

Rios-Marco, P., Martin-Fernandez, M., Soria-Bretones, I., Rios, A., Carrasco, M.P., and Marco, C. (2013). Alkylphospholipids deregulate cholesterol metabolism and induce cell-cycle arrest and autophagy in U-87 MG glioblastoma cells. Biochimica et biophysica acta *1831*, 1322-1334.

Rios-Marco, P., Rios, A., Jimenez-Lopez, J.M., Carrasco, M.P., and Marco, C. (2015). Cholesterol homeostasis and autophagic flux in perifosine-treated human hepatoblastoma HepG2 and glioblastoma U-87 MG cell lines. Biochemical pharmacology *96*, 10-19.

Robbins, E., and Marcus, P.I. (1963). Dynamics of Acridine Orange-Cell Interaction. I. Interrelationships of Acridine Orange Particles and Cytoplasmic Reddening. The Journal of cell biology *18*, 237-250.

Ruiter, G.A., Zerp, S.F., Bartelink, H., van Blitterswijk, W.J., and Verheij, M. (1999). Alkyl-lysophospholipids activate the SAPK/JNK pathway and enhance radiation-induced apoptosis. Cancer research *59*, 2457-2463.

Runge, M.H., Andreesen, R., Pfleiderer, A., and Munder, P.G. (1980). Destruction of human solid tumors by alkyl lysophospholipids. Journal of the National Cancer Institute *64*, 1301-1306.

Sandhoff, K., and Kolter, T. (1996). Topology of glycosphingolipid degradation. Trends in cell biology *6*, 98-103.

Scherf, H.R., Schuler, B., Berger, M.R., and Schmahl, D. (1987). Therapeutic activity of ET-18-OCH3 and hexadecylphosphocholine against mammary tumors in BD-VI rats. Lipids *22*, 927-929.

Schissel, S.L., Keesler, G.A., Schuchman, E.H., Williams, K.J., and Tabas, I. (1998). The cellular trafficking and zinc dependence of secretory and lysosomal sphingomyelinase, two products of the acid sphingomyelinase gene. The Journal of biological chemistry *273*, 18250-18259.

Schnaar, R.L. (2004). Glycolipid-mediated cell-cell recognition in inflammation and nerve regeneration. Archives of biochemistry and biophysics *426*, 163-172.

Schweizer, A., Clausen, H., van Meer, G., and Hauri, H.P. (1994). Localization of O-glycan initiation, sphingomyelin synthesis, and glucosylceramide synthesis in Vero cells with respect to the endoplasmic reticulum-Golgi intermediate compartment. The Journal of biological chemistry *269*, 4035-4041.

Sedmak, J.J., and Grossberg, S.E. (1977). A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Analytical biochemistry *79*, 544-552.

Segui, B., Andrieu-Abadie, N., Jaffrezou, J.P., Benoist, H., and Levade, T. (2006). Sphingolipids as modulators of cancer cell death: potential therapeutic targets. Biochimica et biophysica acta *1758*, 2104-2120.

Semini, G., Hildmann, A., von Haefen, C., and Danker, K. (2014). Glycosidated phospholipids - a promising group of anti-tumour lipids. Anti-cancer agents in medicinal chemistry *14*, 607-617.

Serra, M., and Saba, J.D. (2010). Sphingosine 1-phosphate lyase, a key regulator of sphingosine 1-phosphate signaling and function. Advances in enzyme regulation *50*, 349-362.

Siebzehnrubl, F.A., Reynolds, B.A., Vescovi, A., Steindler, D.A., and Deleyrolle, L.P. (2011). The origins of glioma: E Pluribus Unum? Glia *59*, 1135-1147.

Simons, K., and Gerl, M.J. (2010). Revitalizing membrane rafts: new tools and insights. Nature reviews. Molecular cell biology *11*, 688-699.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.
Singer, S.J., and Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science *175*, 720-731.

Sonnino, S., and Prinetti, A. (2010). Lipids and membrane lateral organization. Frontiers in physiology *1*, 153.

Sonnino, S., and Prinetti, A. (2013). Membrane domains and the "lipid raft" concept. Current medicinal chemistry *20*, 4-21.

Sridharan, S., Jain, K., and Basu, A. (2011). Regulation of autophagy by kinases. Cancers *3*, 2630-2654.
Stancevic, B., and Kolesnick, R. (2010). Ceramide-rich platforms in transmembrane signaling. FEBS letters *584*, 1728-1740.

Staneva, G., Chachaty, C., Wolf, C., Koumanov, K., and Quinn, P.J. (2008). The role of sphingomyelin in regulating phase coexistence in complex lipid model membranes: competition between ceramide and cholesterol. Biochimica et biophysica acta *1778*, 2727-2739.

Stewart, L.A. (2002). Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet *359*, 1011-1018.

Stummer, W., Pichlmeier, U., Meinel, T., Wiestler, O.D., Zanella, F., and Reulen, H.J. (2006). Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. The Lancet. Oncology *7*, 392-401.

Sun, H., Yu, T., and Li, J. (2011). Co-administration of perifosine with paclitaxel synergistically induces apoptosis in ovarian cancer cells: more than just AKT inhibition. Cancer letters *310*, 118-128.

Sun, S.Y. (2010). Enhancing perifosine's anticancer efficacy by preventing autophagy. Autophagy *6*, 184-185.

Tabas, I. (1999). Secretory sphingomyelinase. Chemistry and physics of lipids *102*, 123-130.

Tafesse, F.G., Ternes, P., and Holthuis, J.C. (2006). The multigenic sphingomyelin synthase family. The Journal of biological chemistry *281*, 29421-29425.

Tarnowski, G.S., Mountain, I.M., Stock, C.C., Munder, P.G., Weltzien, H.U., and Westphal, O. (1978). Effect of lysolecithin and analogs on mouse ascites tumors. Cancer research *38*, 339-344.

Tettamanti, G., Bassi, R., Viani, P., and Riboni, L. (2003). Salvage pathways in glycosphingolipid metabolism. Biochimie *85*, 423-437.

Tong, Y., Liu, Y.Y., You, L.S., and Qian, W.B. (2012). Perifosine induces protective autophagy and upregulation of ATG5 in human chronic myelogenous leukemia cells in vitro. Acta pharmacologica Sinica *33*, 542-550.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B., and Simons, M. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science *319*, 1244-1247.

van Blitterswijk, W.J., Klarenbeek, J.B., van der Luit, A.H., Alderliesten, M.C., van Lummel, M., and Verheij, M. (2010). Fas/CD95 down-regulation in lymphoma cells through acquired alkyllysophospholipid resistance: partial role of associated sphingomyelin deficiency. The Biochemical journal *425*, 225-234.

van Blitterswijk, W.J., van der Luit, A.H., Veldman, R.J., Verheij, M., and Borst, J. (2003). Ceramide: second messenger or modulator of membrane structure and dynamics? The Biochemical journal *369*, 199-211.

van Blitterswijk, W.J., and Verheij, M. (2008). Anticancer alkylphospholipids: mechanisms of action, cellular sensitivity and resistance, and clinical prospects. Current pharmaceutical design *14*, 2061-2074.

van Blitterswijk, W.J., and Verheij, M. (2013). Anticancer mechanisms and clinical application of alkylphospholipids. Biochimica et biophysica acta *1831*, 663-674.

van der Luit, A.H., Budde, M., Ruurs, P., Verheij, M., and van Blitterswijk, W.J. (2002). Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. The Journal of biological chemistry *277*, 39541-39547.

Van der Luit, A.H., Budde, M., Zerp, S., Caan, W., Klarenbeek, J.B., Verheij, M., and Van Blitterswijk, W.J. (2007a). Resistance to alkyl-lysophospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin- and cholesterol-deficiency in lipid rafts. The Biochemical journal *401*, 541-549.

van der Luit, A.H., Vink, S.R., Klarenbeek, J.B., Perrissoud, D., Solary, E., Verheij, M., and van Blitterswijk, W.J. (2007b). A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells. Molecular cancer therapeutics *6*, 2337-2345.

van der Sanden, M.H., Houweling, M., Duijsings, D., Vaandrager, A.B., and van Golde, L.M. (2004). Inhibition of phosphatidylcholine synthesis is not the primary pathway in hexadecylphosphocholine-induced apoptosis. Biochimica et biophysica acta *1636*, 99-107.

van Echten-Deckert, G., and Herget, T. (2006). Sphingolipid metabolism in neural cells. Biochimica et biophysica acta *1758*, 1978-1994.

Van Overloop, H., Gijsbers, S., and Van Veldhoven, P.P. (2006). Further characterization of mammalian ceramide kinase: substrate delivery and (stereo)specificity, tissue distribution, and subcellular localization studies. Journal of lipid research *47*, 268-283.

Vaskovsky, V.E., and Kostetsky, E.Y. (1968). Modified spray for the detection of phospholipids on thin-layer chromatograms. Journal of lipid research *9*, 396.

Vescovi, A.L., Galli, R., and Reynolds, B.A. (2006). Brain tumour stem cells. Nature reviews. Cancer *6*, 425-436.

Vink, S.R., Schellens, J.H., van Blitterswijk, W.J., and Verheij, M. (2005). Tumor and normal tissue pharmacokinetics of perifosine, an oral anti-cancer alkylphospholipid. Investigational new drugs *23*, 279-286.

Vink, S.R., van der Luit, A.H., Klarenbeek, J.B., Verheij, M., and van Blitterswijk, W.J. (2007). Lipid rafts and metabolic energy differentially determine uptake of anti-cancer alkylphospholipids in lymphoma versus carcinoma cells. Biochemical pharmacology *74*, 1456-1465.

Vogler, W.R., Berdel, W.E., Geller, R.B., Brochstein, J.A., Beveridge, R.A., Dalton, W.S., Miller, K.B., and Lazarus, H.M. (1996). A phase II trial of autologous bone marrow transplantation (ABMT) in acute leukemia with edelfosine purged bone marrow. Advances in experimental medicine and biology *416*, 389-396.

Vogler, W.R., Berdel, W.E., Olson, A.C., Winton, E.F., Heffner, L.T., and Gordon, D.S. (1992). Autologous bone marrow transplantation in acute leukemia with marrow purged with alkyl-lysophospholipid. Blood *80*, 1423-1429.

Walker, M.D., Alexander, E., Jr., Hunt, W.E., MacCarty, C.S., Mahaley, M.S., Jr., Mealey, J., Jr., Norrell, H.A., Owens, G., Ransohoff, J., Wilson, C.B.*, et al.* (1978). Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. Journal of neurosurgery *49*, 333-343.

Wattenberg, B.W., Pitson, S.M., and Raben, D.M. (2006). The sphingosine and diacylglycerol kinase superfamily of signaling kinases: localization as a key to signaling function. Journal of lipid research *47*, 1128-1139.

Wen, P.Y., and Kesari, S. (2008). Malignant gliomas in adults. The New England journal of medicine *359*, 492-507.

Wieder, T., Orfanos, C.E., and Geilen, C.C. (1998). Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, hexadecylphosphocholine. The Journal of biological chemistry *273*, 11025-11031.

Woolley, P.V., Schultz, C.J., Rodriguez, G.I., Gams, R.A., Rowe, K.W., Jr., Dadey, M.L., Von Hoff, D.D., and McPhillips, J.J. (1996). A phase II trial of ilmofosine in non-small cell bronchogenic carcinoma. Investigational new drugs *14*, 219-222.

Wu, W.K., Coffelt, S.B., Cho, C.H., Wang, X.J., Lee, C.W., Chan, F.K., Yu, J., and Sung, J.J. (2012). The autophagic paradox in cancer therapy. Oncogene *31*, 939-953.

Yao, C., Wei, J.J., Wang, Z.Y., Ding, H.M., Li, D., Yan, S.C., Yang, Y.J., and Gu, Z.P. (2013). Perifosine induces cell apoptosis in human osteosarcoma cells: new implication for osteosarcoma therapy? Cell biochemistry and biophysics *65*, 217-227.

Zhang, L., Lee, K.C., Bhojani, M.S., Khan, A.P., Shilman, A., Holland, E.C., Ross, B.D., and Rehemtulla, A. (2007a). Molecular imaging of Akt kinase activity. Nature medicine *13*, 1114-1119.

Zhang, R., Banik, N.L., and Ray, S.K. (2007b). Combination of all-trans retinoic acid and interferon-gamma suppressed PI3K/Akt survival pathway in glioblastoma T98G cells whereas NF-kappaB survival signaling in glioblastoma U87MG cells for induction of apoptosis. Neurochemical research *32*, 2194-2202.

# ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor Prof. Paola Viani who share with me her expertise and precious time. I feel very grateful for all the fruitful discussions we have gone through together and for her support that allowed me to reach this goal.

I would also like to thank Dr. Paola Giussani for her advice and suggestions.

Then, I wish to thank all my colleagues at the lab, which have been both professional colleagues and friends. A very special thanks goes for Loubna. I will never forget the moral support and encouragement I received from her during my entire experience in the lab. Likewise, I will never forget our mutual observations and our precious times together discussing projects, results and next steps. A deep and sincere thanks also go to Clara and Lory for their advice, suggestions and words of encouragement. Our time together was always very valuable for me both from the professional and personal point of view.

Finally, I will be eternally grateful to my family. Thanks for always being by my side in all the decisions I have taken in my life and for all your patience, understanding and support throughout the toughest moments.