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Perifosine, a novel Akt inhibitor induces apoptosis, cell cycle arrest and has a chemo-sensitizing effect in medulloblastoma cell lines

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PERIFOSINE, A NOVEL AKT INHIBITOR INDUCES APOPTOSIS, CELL CYCLE ARREST AND HAS A CHEMO-SENSITIZING EFFECT IN MEDULLOBLASTOMA CELL LINES

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of common abbreviations

PNET	Primitive Neuro-ectodermal Tumor
CSF	Cerebrospinal Fluid
CSRT	Cranio-Spinal Radiation Therapy
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
GPC	Granular precursor cells
HIC-1	Hypermethylated in Cancer-1
SHH	Sonic Hedgehog
PTCH	Hedgehog receptor Patched
IGF	Insulin-Like Growth Factor 1
MAPK	Mitogen Activated Protein Kinases
STK	Serine Threonine Kinases
ERK	Extra Cellular Signal Regulated Kinases
Cyt C	Cytochrome C
IkB	IkappaB
NFkB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B
PH	Pleckstrin Homology Domain
RD	Regulatory Domain
SH2	Src Homology
PI3K	Phosphatidylinositol 3-OH Kinase
PIP2	Phosphatidylinositol-, 4, 5-Biphosphate
PIP3	Phosphatidylinositol-3, 4, 5-Trisphosphate
PDK1	Phospholipid-Dependent Kinase1
mTOR	Mammalian Target of Rapamycin
PTEN	Phosphatase and Tensin Homologue
MDR	Multidrug Resistance
TNFR1	Tumor Necrosis Factor Receptor1
Mdm2	Murine Double Minute2
ALPs	Alkyl-lysophospholipids

Abstract

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Primary central nervous system (CNS) tumors are the most common solid tumors found in children [35]. While surgery and radiotherapy still remain the standard treatment modalities in pediatric brain tumors, chemotherapy also has an important part in the management of these tumors [87]. However, most of the available chemotherapeutic drugs have suboptimal effectiveness. Deregulation of various pro-apoptotic and anti-apoptotic pathways has been cited as a major mechanism underlying this drug resistance. The role of various serine threonine kinases, including Akt kinases, in promoting drug resistance is being extensively studied in various cancers. A complete understanding of the molecular mechanisms that underlie drug resistance, and the details regarding the specific drug resistance systems operating in medulloblastoma, will help in the development of better therapeutic strategies for these tumors. We have characterized the expression of Akt in medulloblastoma clinical samples and cell lines. The majority of tumor samples and cell lines were found to have elevated endogenous Akt

signaling activity, compared with normal brain samples. Akt kinase activity is involved in cell survival, proliferation and resistance to chemo/radiotherapy in medulloblastoma. In this study, we used a novel drug which has significant activity in suppressing Akt and found that treatment with perifosine led to rapid induction of cell death in medulloblastoma cell lines. Akt inhibitor treatment induced apoptosis and cell cycle arrest. Cell cycle arrest was observed at G1 and G2 cell cycle checkpoints, accompanied by increased expression of the cell cycle inhibitor p21cip1/waf1. We further investigated the involvement of various proteins regulating apoptosis and cell cycle progression in medulloblastoma cells. We also checked the effect of perifosine on regulators of $p21^{waf1/cip1}$, including Akt, MAPK pathways and p53. The effect of perifosine on the MAPK pathway was found to vary with the medulloblastoma cells line studied: for example perifosine treatment increases the activation level of MAPK in VC-312 but had no effect in DAOY cells. On the other hand, perifosine treatment resulted in a decrease in P53 in VC-312 cells without much effect in DAOY cells. Further studies are warranted to check the effect of perifosine on p21waf1/cip1 regulators. Additionally, our studies showed that the combination of perifosine with etoposide or irradiation had a greater than additive effect in DAOY medulloblastoma cells. These studies support an oncogenic role for Akt in medulloblastoma and provide evidence that the Akt inhibition by perifosine, either alone or in combination with other chemotherapeutic drugs, might be an effective therapeutic strategy for the treatment of medulloblastoma.

CHAPTER 1

GENERAL INTRODUCTION

Pediatric Brain tumors and Medulloblastoma

1.1 Pediatric brain tumors and Medulloblastoma

1.1.1 Pediatric Brain tumors

Primary central nervous system (CNS) tumors are the second most common neoplasm after leukemia and the most common solid malignancy of childhood [7]. The prevalence rate of pediatric central nervous system tumors in the United States is estimated at 9.5 per 1000 children [26]. These tumors are the leading cause of death among children ages 0-19 years. Primary brain tumors include astrocytomas (52% of childhood brain tumors), primitive neuroectodermal tumors (PNET) or medulloblastoma (21%), ependymomas (9%), and other gliomas (15%) [7, 35]. These tumors are thought to arise from two main types of brain cells, neurons and glia. Glial cells include astrocytes, oligodendrocytes, ependymal cells, and microglia. The majority of primary brain tumors appear to arise from glial cells (astrocytoma, oligodendrocytoma, ependymoma).

Despite the fact that brain tumors are common solid malignancies in children, the etiology of childhood brain tumors is still largely unknown. An important question to be addressed is: what makes the normal brain cells susceptible to carcinogenesis? There is evidence that the brain is more susceptible to carcinogenesis during prenatal and early postnatal life [7]. During prenatal life there is extensive brain cell growth and proliferation, which lasts longer than in other tissues, thus providing the opportunity for mutagenic changes, leading to tumor

development [7]. In addition, some studies have shown a relative deficiency of DNA repair enzymes in brain tissue. Injections of the DNA damaging agent N [3H]-methyl-N-nitrosourea (10 mg/kg per week) in rats for five weeks led to the accumulation of the mutagenic DNA adduct O^6-methylguanine. The formation of DNA adducts in response to N [3H]-methyl-N-nitrosourea was found to be higher in brain tissue than in other tissues examined, including kidney, spleen and intestine [85]. Moreover, the brain is more sensitive to toxins from the maternal circulation and the environment due to the lack of an efficient blood-CSF barrier during perinatal life [3]. These factors appear to make the brain more susceptible to malignancy as compared to other organ systems during early life.

1.1.2 Medulloblastoma

Medulloblastomas, a common malignant childhood tumor, is classified into two main histological variants, "classical" and "desmoplastic". Other rare variants of medulloblastoma include large cell anaplastic, lipomatous, melanocytic and medullomyoblastoma [64, 70, 119]. The desmoplastic variant has been reported to occur more frequently in one of the cerebellar hemispheres whereas classical medulloblastomas are more frequently located in the vermis of the cerebellum. Histologically, classical medulloblastomas show Homer-Wright rosettes indicating neuronal differentiation whereas desmoplastic medulloblastomas are likely to show astrocytic differentiation [114, 126].

Medulloblastomas are generally associated with a worse prognosis than many other common pediatric cancers, and are thought to arise from cerebellar neural precursor cells [46]. Symptoms associated with medulloblastoma depend upon the age of the patient and tumor location [81]. Adult patients with brain tumors often present with symptoms such as headache,

lethargy, and vomiting due to increased intracranial pressure. Infants with medulloblastoma may present with increasing head circumferences. As medulloblastomas are located in the infra-tentorial fossa, they are also known as infra-tentorial primitive neuro-ectodermal tumors (iPNETs). The supra-tentorial PNETs can be differentiated clinically from infra-tentorial PNETs, as these patients often present with seizures, endocrinopathies or visual deficits which are not commonly seen in iPNETs [81].

1.2 Treatment of Medulloblastoma

Therapeutic options for medulloblastomas include surgery, chemotherapy and radiation therapy. These treatment modalities commonly have grave side effects, including memory-, attention-, motor function-, language- and visuospatial deficits [107].

1.2.1 Surgical resection

Surgical resection of medulloblastoma is an essential part of treatment that has led to improved survival in children with localized disease. There are sophisticated neurosurgical techniques that minimize neurological deficits and improve the survival and quality of life in patients with brain tumors [67, 73, 94]. Complete surgical resection is usually performed for localized tumors. However, a subtotal resection is performed if the tumor is invading the brainstem. The strongest prognostic indicator for survival of patients with medulloblastoma is the degree of surgical resection of the tumor [143]. Surgical resection may also be associated with complications, including ataxia, hemiparesis, sixth cranial nerve palsy and cerebellar mutism [72]. The cerebellar mutism is characterized by a transient decrease in speech output. It is a unique complication associated with extensive surgery and thought to result from excessive dissection at the junction of the cerebellar peduncles and the brainstem [112].

1.2.2 Radiation therapy

While surgery is the ideal first-line treatment for medulloblastoma, some tumors are not amenable to resection because of their location- and extension to vital and delicate brain structures and the grave side effects associated with surgery in such areas. Medulloblastomas frequently disseminate through the cerebrospinal fluid (CSF) and this makes the tumor inaccessible for surgical resection [10, 147]. So cranio-spinal radiation therapy (CSRT) is an essential part of the management in many pediatric brain tumors. The standard CSRT dose ranges from 36Gy to 54Gy, depending upon the location of the tumor. The combination of radiation therapy along with surgical resection has improved the 5 year progression free survival in patients with medulloblastoma to 50-60 % [1, 10].

Another challenge in treating medulloblastoma is a high rate of recurrence. Studies performed using different doses of radiation therapy have demonstrated that there is a higher rate of tumor recurrence (approximately 50–70%) in medulloblastomas treated with lower doses of radiation (36 Gy in 20 fractions to 23.4 Gy in 13 fractions) [31], however a higher dose of radiation therapy (54Gy) is associated with more side effects such as long-term intellectual- and learning deficits as well as growth hormone deficiencies [1]. Combining chemotherapy with low dose radiotherapy is equally effective in reducing recurrence of medulloblastoma compared to higher doses of radiation therapy [54].

1.2.3 Chemotherapy

Chemotherapy is often used as an adjunct to surgery and radiation therapy. Common chemotherapeutic drugs used in medulloblastoma treatment and extensively studied are 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), cisplatin, and vincristine [54]. These drugs have high lipid solubility, can easily penetrate the blood brain barrier and are used along with lower

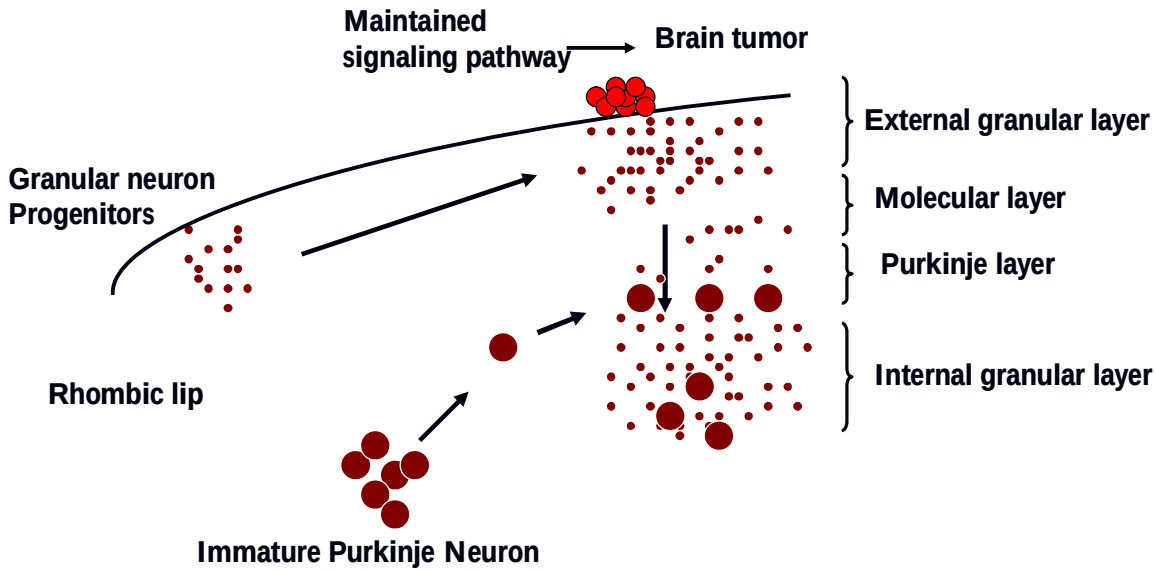
doses of radiation (36Gy). The combination of surgery along with radiation and chemotherapy is used as the new standard for treatment in children with medulloblastoma [54]. Despite the use of all this combination therapy, there has not been much progress in the five-year survival of afflicted individuals. This indicates a need for novel treatment approaches. Studies done on medulloblastomas have revealed that there are specific genetic alterations associated with these tumors, and which are responsible for formation of these tumors [2, 22, 60]. These genetic alterations include either over-expression of a tumor promoting protein (oncogene), or a decrease in expression or loss of activity in tumor suppressor proteins. Generally a combination of these alterations leads to neoplastic transformation [38]. Understanding the events of molecular pathogenesis in medulloblastoma would help in designing therapies to target these altered genes and prevent the transformation of normal cells to tumor cells.

1.3 Molecular biology and cell signaling in Medulloblastoma

1.3.1 Molecular biology of medulloblastoma

There has been considerable progress in the knowledge of molecular pathogenesis of pediatric brain tumors [57, 66]. These tumors arise due to a disorder in cerebellar development. The normal development of the cerebellum is predominantly postnatal [47]. It involves two main populations of multipotent neural progenitor cells, granular precursor cells and immature Purkinje cells [8] (Figure 1.1). Granular precursor cells migrate from the rhombic lip of the diencephalon to form the external granular layer. Here these cells undergo proliferation and then migrate to form the internal granular layer, where they exit the cell cycle and differentiate into mature granule cells. Migration and differentiation of these cells is maintained by cell signaling pathways via interaction with Purkinje cells. Disruption of normal signal transduction impairs

Figure 1.1 Role of cell signaling pathways in medulloblastoma development. Aberrant activation of cell signaling pathways including Sonic Hedgehog (SHH) signaling and serine threonine kinases (STKs) leads to rapid proliferation of granule cell precursors (GPC) in external granular layer cells and limits the ability of GPC cells to migrate and terminally differentiate to form internal granular layer. The hyper-proliferation of GCP in external granular layer is believed to be responsible for tumor formation (medulloblastoma).



migration and differentiation leading ultimately to hyper proliferation in the external granular layer and eventually tumor formation (medulloblastoma). Medulloblastomas have been found to exhibit chromosomal loss and gene amplifications [100]. Two main common regions of genetic loss have been observed on chromosomes 17 and 9q [23]. Loss of 17p12-3-1 is found in 50% of medulloblastomas - most commonly in the classical medulloblastoma subtype [22]. Loss of 9q is common in other subtypes of medulloblastoma, including nodular or desmoplastic medulloblastoma [116]. The phenomenon of loss of one chromosome, segment, or allele is called loss of heterozygosity. There is loss of normal function of one allele, while the other allele has already been inactivated [115]. A commonly altered tumor suppressor gene on chromosome 17p is TP53. Thus, it is possible that loss of heterozygosity on chromosome 17p might be associated with mutation of p53 in medulloblastoma. However, p53 mutation is less frequent in medulloblastoma (10%) [22] than loss of heterozygosity on chromosome 17p. In fact, it has been shown that the locus of loss of heterozygosity is more distal (17pl3.3) on chromosome 17 than the locus of p53 (17pl3.1) [22]. These results imply that there are other gene candidates on 17p that might be responsible for the pathogenesis of medulloblastoma. Another gene located on chromosome 17p13.3 is HIC-1 (Hypermethylated in cancer), which has been shown to be epigenetically silenced in medulloblastoma. Hyper-methylation of the HIC-1 gene promoter has been detected in 85% (33/39) of medulloblastoma biopsies and in 88% (7/8) of medulloblastoma cell lines [141]. This finding suggests that medulloblastomas exhibit reduced HIC-1 expression by hyper-methylation and this gene might have tumor suppressor functions.

Several other genes involved in embryonic brain development are also found to be altered in medulloblastoma. The human homologue of the Drosophila segment polarity gene encoding Patched (PTCH) has been found to be mutated in medulloblastoma [103]. Sonic Hedgehog (SHH)

signaling is a key regulator of embryonic development, which regulates cell proliferation and cell fate in neural precursors in the nascent cerebellum. SHH ligand binds to the hedgehog receptor Patched (PTCH) and relieves Patched-mediated inhibition of another receptor, Smoothened (SMO). Smoothened activates a SHH mediated cell signaling pathway [48]. Inactivating mutations in the PTCH gene have been found in medulloblastoma (MB). Screening of medulloblastoma tumor samples using single-strand conformational polymorphism analysis revealed non-conservative PTCH mutations in 27% of the desmoplastic variant MB tumors [103], suggesting that the PTCH gene is involved in the development of the desmoplastic variant of MB. To investigate the role of PTCH signaling in medulloblastoma formation, mouse models have been generated, that carry deletions of genes in the Shh pathway. The majority of PTCH mutant animals did not develop medulloblastoma tumors [131], suggesting activation of Hedgehog signaling alone is not sufficient for medulloblastoma induction. There is also evidence suggesting the involvement of additional signal transduction pathway components in medulloblastoma tumor formation [105]. Induction of insulin-like growth factor (IGF) signaling along with PTCH mutation in one report enhanced tumor formation from 15% to 39% [105]. Insulin-like growth factor (IGF) acts on its cognate extra cellular receptors, such as IGF1-R, which leads to activation of serine threonine kinases (STKs), including mitogen activated protein kinases (MAPKs) and the Akt pathway. Studies done in our laboratory showed increased expression of Akt kinase in medulloblastoma clinical samples, as compared to normal cerebellum. Activation of STK-mediated cell signaling pathways is likely to be an important mechanism in medulloblastoma pathogenesis.

1.3.2 Cell signaling in medulloblastoma

Serine threonine kinases are the family of protein kinases that phosphorylate the hydroxyl (OH) group of serine and threonine residues in their substrates. These kinases include mitogen activated protein kinases (MAPKs), protein kinase A (PKA), Akt kinases (protein kinase Bs) and protein kinase C isotypes (PKC) [12, 23]. This review will focus on MAPK and Akt (PKB). The MAPK family includes extra cellular signal regulated kinases (Erk), p38 MAPK and c-Jun N-terminal kinase (JNK). The MAPKs play a critical role in the transmission of growth promoting and growth inhibiting signals from cell membrane receptors to the signal mediators in the cytoplasm and nucleus, thereby altering gene expression. This way MAPKs play an important role in maintaining the balance between cell survival and programmed cell death (apoptosis). In general, the JNK and p38 pathways usually promote apoptosis as a response to cellular stress, whereas the ERK pathways inhibit apoptosis [23]. Various proteins involved in the MAPK pathway are mutated or aberrantly expressed in many human cancers [18, 40].

Akt, also known as PKB, is another kinase involved in the regulation of cell survival [25]. The role of Akt has been well established in the regulation of apoptosis [96]. Apoptosis involves a series of biochemical events that lead to activation of cysteine-aspartic acid proteases (caspases). These active caspases have proteolytic activity and play an essential role in apoptosis [30]. Based on the level at which caspases they are activated in the pathway, they can be categorized as initiator caspases (caspase 8, caspase 9) and executor caspases (caspase 3 and 7) [30]. Initiator caspases are activated either by the mitochondria via the intrinsic pathway (caspase-9) or by cell surface receptors via the extrinsic pathway (caspase-8). Activation of the intrinsic pathway of apoptosis involves release of cytochrome C across outer mitochondrial membrane. This release depends on outer mitochondrial membrane potential which is regulated by proteins Bad (pro-apoptotic) and Bax (anti-apoptotic). Increased levels of Bad induce mitochondrial membrane

Figure 1.2 Regulation of apoptosis by the Akt pathway. The Akt pathway regulates the intrinsic pathway of apoptosis. Activation of this apoptotic pathway depends on mitochondrial release of cytochrome C. Release of cytochrome C activates caspases 9, which in turn activates caspases 7 and caspase 3. These active caspases cleave target proteins and execute apoptosis. Release of cytochrome C is tightly regulated, and is dependent on the outer mitochondria membrane potential. This membrane potential is regulated by proteins like Bax. Increased activity of Bax protein increases the release of cytochrome C form mitochondria. Akt directly phosphorylates Bax and inhibits its activity. Akt also phosphorylates caspase 9 and inhibits its proteolytic activity leading to inhibition of caspase 3 and caspase 7activation.

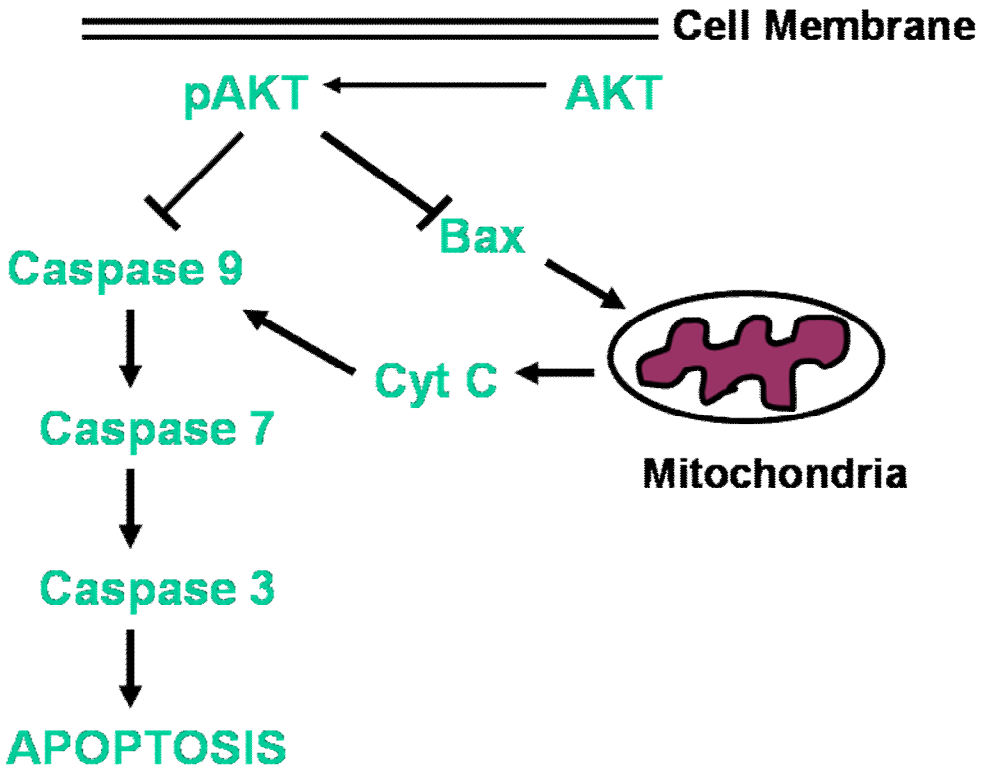


Figure 1.2

permeability to cytochrome C. Cytochrome C then binds to apoptosis protease-activating factor (APAF), forming the apoptosome and activates caspase-9 [33]. Akt mediated cell signaling has been shown to directly inhibit the mitochondrial induced caspase activation pathway by phosphorylating caspase-9 (Figure 1.2), thus inhibiting its function [23]. Akt can also directly phosphorylate Bad (Figure 1.2) and inhibit its function, thereby stabilizing the mitochondrial membrane [33]. This indicates that Akt has a role in inhibiting intrinsic pathway of apoptosis.

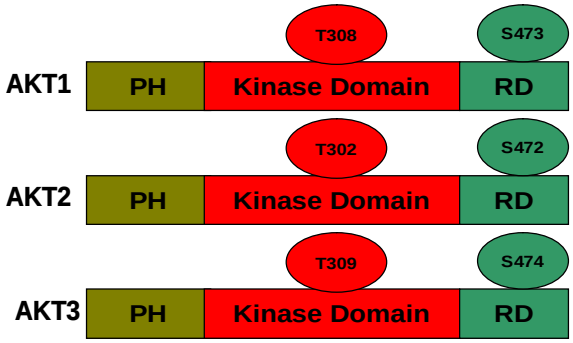
The extrinsic apoptotic pathway mediated by the cell membrane includes activation of receptors present on cell membrane (death receptors). These death receptors include Fas (CD95), Tumor Necrosis Factor Receptor 1, 2 (TNFR1, TNFR2) and TNF-related Apoptosis Inducing Ligand (TRAIL) Receptor. The Fas ligand binds to receptors on the cell surface and recruits the death domains to these receptors. The recruitment of death domain to these receptors activates caspase 8, which further triggers activation of caspase 3 or 7 [58]. Inhibition of Akt has been shown to activate extrinsic pathway of apoptosis [95], suggesting its role in extrinsic apoptotic pathway.

Akt also regulates cell proliferation through the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling. Akt directly phosphorylates and inactivates IkappaB (IkB), which is the endogenous inhibitor of this NFkB –mediated proliferative signaling. Akt mediated phosphorylation of this protein leads to nuclear translocation of NfkB and transcriptional activation of its target genes [29].

1.4 Cell signaling and Serine/threonine kinases

1.4.1 Akt (Protein kinase B)

Figure 1.3 Schematic diagrams of the Akt isoenzymes. The Akt kinases contain conserved domain structures including N-terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl–terminal hydrophobic regulatory domain (RB). The protein has two important phosphorylation sites in the kinase domain and in the regulatory domain. The table shows the degree of structural homology among these three Akt isoforms.



PH = Pleckstrin homology domain

RD = Regulatory Domain

Table 1.1 Overall homology of primary structure of Akt enzymes.

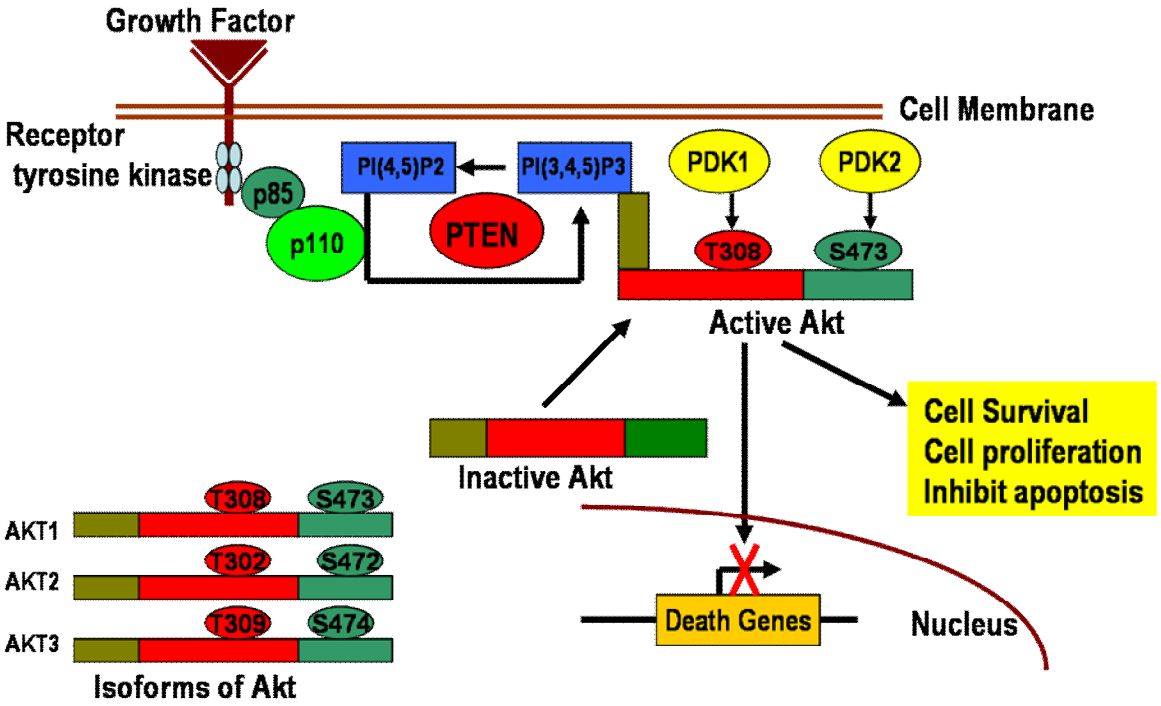
Akt Isoforms	Degree of structural homology
Akt 1 and Akt 2	81%[77]
Akt 1 and Akt 3	83%[77]

Akt, also called protein kinase B (PKB), is a member of the serine/threonine kinase family. As mentioned above, Akt plays an important role in the regulation of mammalian cell signaling pathways. The first genetic sequence of Akt was described in the retrovirus, Akt8. The virus containing this gene was able to transform tissue culture cells into tumor-like cells [128]. There are three isoforms of Akt [Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ)]. These isoforms are encoded by different genes and differentially expressed by cells. They have 80% homology in their structure but vary in their functions [25, 40]. Akt1 (PKBα) is ubiquitously expressed by all cells, whereas Akt2 (PKBβ) is predominantly expressed in insulin sensitive cells, including skeletal muscle, adipose tissue and the liver. Akt2 has been shown to be involved in glycogen synthesis and glucose uptake in these cells [40, 71]. The expression of Akt3 is restricted to the brain and testes. Despite differing isotype expression by cells, all Akt isotypes share amino acid sequence homology in their structure in humans. Akt proteins have three conserved domains: a pleckstrin homology domain (PH), a kinase domain and a regulatory domain (RD) (Figure 1.3). The N-terminus of the Akt protein contains the PH domain, which is connected to the kinase domain in the middle by a hinge region. The pleckstrin homology domain is involved in protein-protein and protein-lipid interactions. The kinase domain is specific for phosphorylating serine/threonine residues in target proteins. The C-terminal region of Akt is necessary for the induction and maintenance of its kinase activity [40]. The three Akt protein kinase isoforms have distinct functions in the regulation of metabolism, cell growth, and apoptosis [50]. The mechanisms by which their signaling specificity is achieved remain largely unclear. All Akt isotypes have the same mechanism of activation because of their structural similarity.

1.4.2 Mechanism of Akt activation

Activation of all Akt isotypes involves the cell membrane (Figure 1.4). The binding of

Figure 1-4. Schematic presentation of the mechanism of activation of Akt. Binding of growth factors to RTKs activates PI3K which triggers the production of PIP3 from PIP2 at the plasma membrane. PIP3 then interacts with the pleckstrin homology domain of Akt. Interaction between Akt and PIP3 at the cell membrane induces the translocation of Akt from cytosol to the membrane. At the cell membrane Akt is in close proximity with PDK1 and PDK2 and is phosphorylated at threonine-308 and serine-473 sites respectively. Phosphorylated Akt becomes active and is involved in cell survival, cell proliferation and inhibition of apoptosis.



growth factors, e.g. IGF, to their receptor tyrosine kinase (RTK) on the cell membrane leads to auto-phosphorylation of tyrosine residues, which are present in the intracellular region of these receptors. Intracellular proteins containing Src homology (SH2) domains bind to these sites of tyrosine phosphorylation [52]. One such protein containing the SH2 domain is phosphatidylinositol 3-OH kinase (PI3K). PI3K contains an 85 kDa regulatory subunit (which consists of a SH2 domain) and a 110 kDa (p110) catalytic subunit (kinase domain). The regulatory subunit (p85) of PI3K binds to the receptor tyrosine kinase. Binding of the regulatory unit to receptor activates the catalytic subunit (p110) of PI3K [37]. This active kinase, p110, then catalyzes the conversion of phosphatidylinositol-, 4, 5-biphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) in the cell membrane. PIP3 then recruits proteins containing pleckstrin homology domain (PH) to the membrane, including Akt and phospholipid dependent kinase1 (PDK1) [37]. Binding of PDK1 to PIP3 leads to autophosphorylation (activation), which in turn phosphorylates Akt at threonine (Thr^{308}) [33]. Presence of phosphate at Thr^{308} recruits other kinases for phosphorylation at serine (Ser^{473}). The Ser^{473} is present in the carboxyl-terminal hydrophobic motif of Akt. The mechanism of Ser473 phosphorylation remains unclear. It is thought that Ser473 is phosphorylated by phospholipd depend kinase2 (PDK2). Recent studies have shown that Ser473 is phosphorylated by Mammalian Target of Rapamycin (mTOR) in association with the protein Rictor [113]. The conversion of PIP2 to PIP3 is tightly regulated. There are several phosphatases including phosphatase and tensin homologue (PTEN), SHIP1 and SHIP2, which convert PIP3 back into PIP2. A decrease in the function of these phosphatases leads to accumulation of PIP3, which recruits and up-regulates Akt-mediated cell signaling. The inactivation of PTEN by mutations is

Table 1.2 Phosphorylation Targets of Akt*

Substrate	Phosphorylation site	Effect of Akt-mediated Phosphorylation
Apoptotic Markers		
Pro-caspase 9	Ser196	Suppression of caspase-9-induced cell death
BAD	Ser136	Association of BAD with 14-3-3 proteins; Suppression of BAD-induced cell death
IKK (IκBkinase)	Thr23	Induction of NF-□B transcriptional activity
Forkhead family (FKHR,FKHRL1, AFX)	Thr24, Ser256, Ser319 (FKHR) Thr32, Ser253, Ser315 (FKHRL1) Thr28, Ser193, Ser258 (AFX)	Association with 14-3-3 proteins; prevention of transcription of proapoptotic Genes
Cell cycle		
$p21^{waf1/cip1}$	Thr145	Cell cycle progression
MDM2		Increased p53 ubiquitination, degradation Suppression of p53 activity
Other transcriptional/ translational Regulation		
CREB	Ser133	Increased transcription of CREB-regulated survival genes
mTOR/FRAP	Thr2446, Ser2448	Modulation of mRNA translation
AR	Ser210, Ser790	Decreased transcription of AR-regulated genes; modulation of AR-mediated apoptosis
TSC1, TSC2		Attenuated inhibitory effect of TSCs on mTOR
eNOS		Activation of eNOS; production of nitric oxide
Telomerase	Ser227, Ser824	Enhanced telomerase activity

Modified from Curr Cancer Drug Targets. 2004; 4(3):235-56

commonly involved in solid tumor formation [90]. Activated Akt catalyzes the phosphorylation of target proteins containing the minimum sequence motif of R-Xaa-R-Yaa-Zaa-S/T-Hyd, where Xaa is any amino acid, Yaa and Zaa are small residues other than glycine and Hyd is a bulky hydrophobic residue (Phe, Leu) [4]. The Akt target proteins are involved in regulation of various functions, including apoptosis and cell cycle regulation. Phosphorylation of these target proteins changes their functional properties and promotes cell survival and proliferation [90]. Table 1.2 lists many of the well-documented Akt targets and their functional roles in cells.

1.4.3 Akt signal transduction and role in tumor formation

Aberrant activation of the Akt pathway is a common underlying factor in the pathogenesis of human malignancies. Activation of Akt in tumor cells can occur by amplification of its genes or by activation of its up-stream regulators. Amplification of Akt1 gene (up to 20 fold) has been found in 20% of gastric adenocarcinomas [122]. Similar to Akt1, Akt2 was also found amplified in approximately 10% of pancreatic carcinoma cell lines and pancreatic tumor specimens [19]. Treatment of these pancreatic carcinoma cells with antisense Akt2 RNA markedly reduced the tumorigenicity in nude mice supporting a functional role of Akt2 in tumorigenicity [19]. But the incidence of gene amplification of Akt isoforms is far less than aberrant activation of Akt signaling in tumors. This suggests that there might be deregulation of up-stream proteins that regulate Akt signaling.

The upstream regulators of Akt signaling include growth factor receptors, Ras protein, PTEN and Phosphoinositide Kinase-3 (PI3K). Mutation of Ras protein has been shown to mediate tumor transformation by activation of the Akt pathway [108]. Mutations of PTEN, a negative regulator of Akt signaling, are also common in tumors [124, 60]. Reduction of PTEN expression was found to be associated with PTEN promoter hyper-methylation in 50% of the

medulloblastoma tumor samples [60]. We have shown that Akt isoforms are increased in medulloblastoma clinical samples as compared to normal brain. The aberrant activation of Akt signaling in medulloblastoma, thus, might be because of amplification of Akt isoform genes or by alteration in Akt regulatory proteins.

1.5 Cell signaling and chemo-resistance

Chemo-resistance- that is, resistance of tumor cells to chemotherapeutic drugs- is another challenge in tumor therapeutics. One of important chemo-resistance is multidrug resistance in which tumor cells are resistance to a broad range of structurally and functionally unrelated drugs [57, 58]. Generally, chemotherapies kill tumor cells by causing DNA damage, which activates death inducing cell signaling pathways. However, some tumor cells possess mutations in these cell signaling pathways. These tumor cells are not capable of inducing apoptosis in response to chemotherapy and therefore are resistant to it [30].

Since cytotoxic drugs kill tumor cells via apoptosis, induction of apoptosis is one of the main predictors of response to chemotherapy. Alteration in the expression of genes that regulate apoptosis can affect the chemo-sensitivity of tumor cells leading to resistance to chemotherapies. This type of chemo-resistance is termed apoptosis negative multidrug resistance (–MDR) [134]. Since the ability of a cell to undergo apoptosis is an intrinsic property, it appears that apoptosis-negative multidrug resistance can mediate a much wider range of drug resistance than any of the other molecular resistance mechanisms [13]. It is well known that alterations in the genes involved in apoptosis like P53, Bcl-2, Ras have an important role in carcinogenesis. Much research has been done to study the effect of alterations in the expression of various pro-apoptotic and anti-apoptotic factors on sensitivity of tumor cells to drugs. A study on pediatric glial tumors showed that cytotoxic effects of Doxorubicin and Actinomycin D correlated with Bax expression [89]. In

another study conducted with glioblastoma cells, it was shown that as drug resistance of these cells increased, the expression of anti-apoptotic Bcl-2 and Bcl-xl increased and expression of pro-apoptotic Bax decreased [127]. In addition, a role for proto-oncogenes like Ras and c-Jun in protecting the tumor cells from apoptosis has been suggested [14, 102]. Thus, it is clear that further studies are required to determine the role of apoptotic pathways in mediating drug resistance and whether targeting these apoptotic pathways can be successful in improving the efficacy of chemotherapy.

1.5.1 Akt (PKB) and chemo-resistance

As mentioned previously Akt is known to promote cell survival [21, 25]. The role of PI3K/Akt in human medulloblastoma cell lines has been studied and it has been shown that PI3K/Akt pathway is important in medulloblastoma cell growth. Activation of this pathway is associated with decreased expression of PTEN in these cells has been found to contribute to chemo-resistance [60]. Chemotherapy (paclitaxel and doxorubicin) induced cell death can be enhanced by inhibition of Akt signaling [65]. In a study using ovarian cancer cells, it was found that constitutively active Akt (CA-Akt) renders a cisplatin-sensitive ovarian carcinoma cell line resistant [148]. The mechanism by which CA-Akt rendered these cells resistant was reported to be through inhibition of the mitochondrial/intrinsic pathway of apoptosis induced by cisplatin. Clark et al. studied the role of PI3K/Akt pathway in breast cancer chemo-resistance [21]. They studied six breast cancer cell lines, four of which had constitutively active Akt [75]. Inhibiting the PI3K/AKT with LY294002 (an AKT inhibitor) and dominant negative mutant Akt enhanced the induction of apoptosis by chemotherapy (doxorubicin, trastuzumab and tamoxifen). These studies show that Akt activity promotes chemotherapeutic resistance. Knockout of Akt isoforms using siRNA in cancer cells also increased their sensitivity to cisplatin, suggesting that Akt

isoforms are involved in chemo-resistance in these cells [42]. In addition to apoptosis, the p53-murine double minute (mdm2) pathway was also found to be involved in Akt mediated chemo-resistance. Chemotherapy induces DNA damage which activates P53, and p53 activation increases the expression of proteins involved in apoptosis. Mdm2 negatively regulates p53, and Akt regulates the level of mdm2. In addition Akt may be directly involved in sensing the DNA damage and affecting the kinases or proteins involved at DNA damage check points [63]. These studies indicate that the PI3K/Akt pathway plays an important role in chemo-resistance.

In summary, AKT, which was found to be over-expressed in medulloblastoma cells, clearly has a very important role to play in the development of medulloblastomas and can enhance cell proliferation by its inhibitory effects on apoptosis. Suppression of Akt signaling by genetic or pharmacological means may, therefore, have therapeutic implications [129].

1.5.2 Other Cell signaling pathways and chemo-resistance

1.5.2.1 MAPK and chemo-resistance

MAPK is part of the mitogen activated protein kinase family. This kinase is activated after stress and chemotherapeutic insults, and promotes apoptosis. Activated JNK (member of MAPK) phosphorylates transcription factors such c-Jun and ATF2 which then bind to AP-1 and increase FasL transcription. Increased FasL transcription induces caspase activity, leading to apoptosis. Chemotherapy-induced JNK activity has been studied in ovarian carcinoma cell lines [84]. These cell lines were treated with cisplatin at different time points. JNK activity against GST-c-Jun protein and phosphorylated JNK and p38 levels on western blots were found to be higher in the cisplatin-sensitive cell line. Ovarian carcinoma cell lines sensitive to cisplatin also showed prolonged activation of JNK/ P38 whereas cisplatin-resistant cell lines were found to show only transient activation of JNK/P38. This differential activity was thought to be

responsible for chemo-sensitivity, indicating the role of these kinases in cisplatin-induced death. For further confirmation, the authors used c-Jun -/-3T3 fibroblasts and inhibited JNK with a pyridinylimidazole compound SB202190, and found that inhibiting these kinases made cisplatin-sensitive cells resistant [84]. These findings support the notion that one of the key determinants of chemotherapy-induced apoptosis is the duration of JNK and P38 activation, and defects in this pathway may contribute to chemo-resistance.

1.5.2.2 P53 and chemo-resistance

Most chemotherapeutic agents act by damaging DNA, activating cell signaling pathways that lead to cell death. One of the main mediators of the DNA damage response is the tumor suppressor gene P53 [119]. The exact mechanism by which DNA damage activates P53 is unknown but it is hypothesized that DNA damage leads to specific post-translational modifications of P53 [121]. Phosphorylation is one potential mechanism that regulates the activity of P53. *In vitro* studies have identified multiple sites of phosphorylation within the amino- and carboxyl- terminal (Serine6, 15, 33) of P53. The main kinases identified in the phosphorylation of p53 at Serine 6, 33) are ATM and DNA-PK [121]. The source of phosphorylation in p53 at ser 15 is debatable. While some studies implicate ATM phosphorylase[119], cells from patients with ataxia telangiectasia (and are consequently deficient in ATM) also showed phosphorylation at p53 at ser 15 upon DNA damage. This suggests that ATM is not absolutely required for phosphorylation [121]. Phosphorylation of P53 increase its ability to trans-activate its target genes. It also decreases P53 degradation (ubiquitination) by decreasing its interaction with mdm2. The duration and severity of DNA damage determines the fate of cell whether to undergo p53 mediated cell cycle arrest or apoptosis. Short duration and less severe damage results in cell cycle arrest and cell repair by p53-mediated increase in expression of the proteins $p21^{cip1/waf1}$ and

GADD45. Prolonged and severe damage, on the other hand, leads to induction of apoptosis through the mitochondrial death pathway by up-regulating proteins like Bax and Fas [41].

1.6 Targeting cell signaling pathway and Alkyl phospholipids (ALPs)

As mentioned above, significant progress has been made in treatment of pediatric brain tumors through advances in neurosurgery, radiotherapy and chemotherapy. Despite these measures, the five-year survival is still low and there are significant long-term sequelae associated with these therapies. With the expansion in knowledge of pediatric tumor biology, new genes and proteins involved in tumor survival and proliferation are established as being over-expressed in these tumors. Targeting these molecules (molecular targeted therapy) may augment the effect of conventional therapies or even replace them.

1.6.1 Overview

Most of the available chemotherapeutic agents target DNA, thus affecting cell division and proliferation. Lack of selectivity is the major problem with these chemotherapeutic drugs as these affect even normal tissues with high proliferation rates including bone marrow (leading to anemia, leucopenia, infection, thrombocytopenia bleeding), the gastrointestinal epithelial cells (diarrhea, vomiting, nausea, surface ulcerations), and the cells of the hair follicles (alopecia) [43]. Alkyl-lysophospholipids (ALPs) are synthetic ether-linked analogues of lysophosphatidylcholine lipids that have been found to have promising anticancer activity and minimal effect on normal cells. The prototype of these ALPs is eldofosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine). Perifosine (octadecyl-[N, N-dimethyl-piperidinio-4-yl]-phosphate) is clinically the most advanced Akt inhibitor and is structurally related to eldofosine. It is orally bioactive, has an improved tolerability profile and has been reported to exert a strong anti-neoplastic effect in human tumor cell lines [139]. Perifosine is currently being tested in phase II clinical trials for

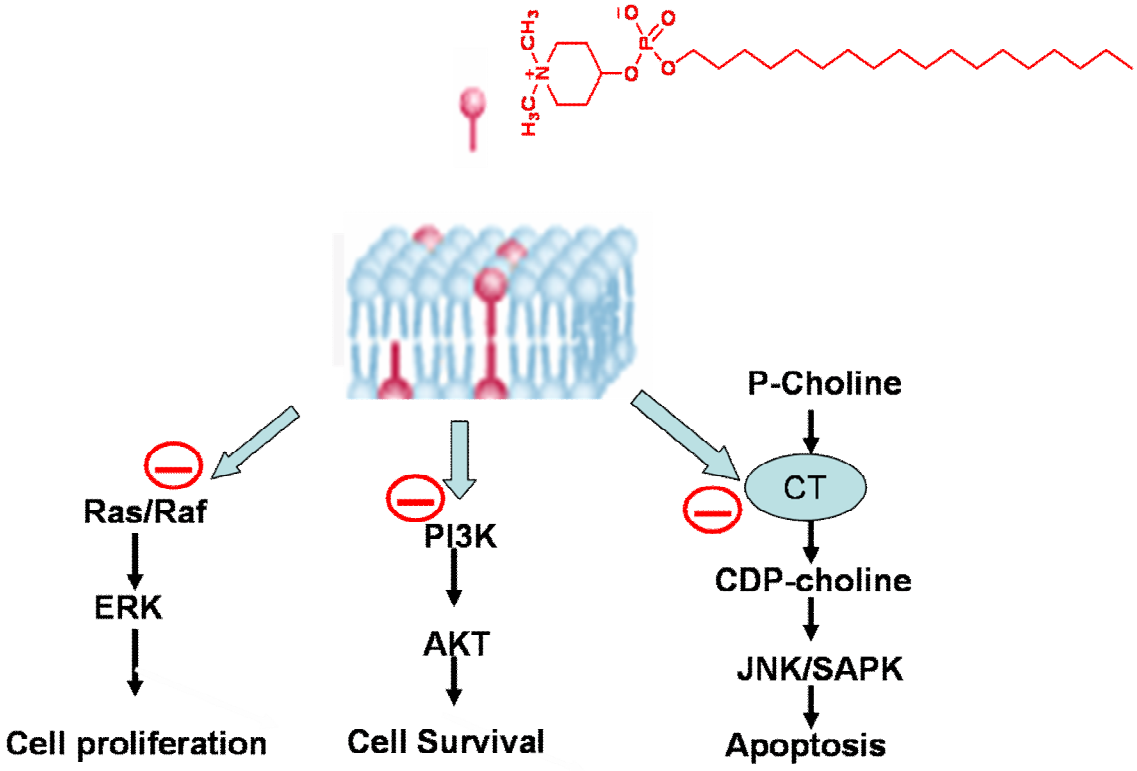
treatment of human cancers [74]. It has a long half-life as it is not inactivated by acyltransferase into lecithin (phosphatidylcholine, PC) or conversion by lysophospholipase in to glycerophosphocholine [43].

1.6.2 Mechanism of action of Alkyl phospholipids (ALS)

The mode of action of Alkyl phospholipids (ALS) primarily involves interfering with turnover of phospholipids. ALS or phospholipids are internalized into cells by raft-mediated endocytosis and phospholipids translocase [91]. They then interfere with choline cytidyltransferase (CT) (figure 1.6), one of the main enzymes for phosphatidylcholine synthesis [6]. This leads to inhibition of lipid turnover in cell membrane and therefore inhibition of lipid dependent cell signaling pathways in tumor cells (Figure 1.6). Akt and MAPK are the two main cell signaling pathways which require lipid moieties as substrates for their activation [69]. Treatment with perifosine has been shown to affect the phosphorylation status of Akt on Ser473 and Thr308 in prostate cancer cells within 30 minutes, without affecting the total amount of Akt protein. Perifosine interferes with phosphorylation of Akt by decreasing its translocation to the plasma membrane and thereby decreasing the association of Akt and PDK1, which is critical for Akt activation [69]. The MAPK pathway is another cell signaling system that is affected by ALS. The effect of perifosine on pErk varies from cell to cell. Usually, perifosine treatment leads to a decrease in pErk levels, but in some cells including HaCaT human keratinocyte cells, perifosine treatment activates Erk [28]. No studies have been conducted as yet, to determine how perifosine might affect pErk. One possible mechanism by which perifosine may increase pErk is through

Figure 1.5 Mechanism of action of alkylphospholipids, perifosine*.

There are two structural moieties in perifosine (shown in this figure), the polar head group and the non-polar alkyl chain. Perifosine is internalized into cell membranes, since the nonpolar group is hydrophobic in nature. It then interferes with lipid turnover by inhibiting choline cytidyltransferase (CT). CT is the main enzyme for lipid (phosphatidylcholine) synthesis, which is a major constituent of cell membranes, and also plays a role in membrane-mediated cell signaling [140]. Inhibition of lipid turnover interferes with Akt and MAPK activation. Inhibition of Akt and MAPK affects cell survival and proliferation. Perifosine also activates the stress signaling pathway JNK/SAPK, leading to apoptosis.



* Modified from http://www.aeternazentaris.com/docs/products/img/en/3.perifosine1.gif

Ras, which is inhibited by pAkt. Perifosine treatment decreases pAkt which attenuates this inhibition and might therefore increase the levels of pErk.

In summary Akt has a protective role in cellular apoptosis, hence, targeting Akt is a novel approach to overcome the apoptotic resistance in medulloblastoma. Akt has been found to be over-expressed in a number of human tumors including medulloblastoma. This project was designed to check the efficacy of perifosine on tumor cell survival. We also sought to determine whether this Akt inhibitor could be used as an adjuvant therapy for medulloblastoma in combination with conventional therapy.

The following specific aims were proposed:

1. To characterize Akt isotypes in medulloblastoma and examine the role of Akt activity on cell viability in medulloblastoma cell lines by attenuating Akt signaling pharmacologically.

2. To determine if inhibition of Akt leads to a decrease in chemo/radio-resistance in medulloblastoma cell lines.

3. To determine whether the mechanism of action of cell death following Akt down regulation in medulloblastoma cell lines involves the apoptotic pathway.

4. To examine the effect of perifosine on cell cycle arrest in medulloblastoma.

This study showed that treatment of medulloblastoma cells with the Akt inhibitor, perifosine led to rapid induction of cell death in medulloblastoma cell lines, with pronounced suppression of phosphorylated Akt in a time- and concentration- dependent manner. The mechanism of cell death involved apoptosis, as indicated by cleavage of caspases. Combining single dose treatment regimens of perifosine with sub-lethal doses of etoposide or irradiation which are currently used in treatment of these lethal tumors) demonstrated a greater than additive effect in medulloblastoma cells DAOY. Low dose (15 μM) perifosine induced cell cycle arrest

at the G1 and G2 cell cycle checkpoints, accompanied by increased expression of the cell cycle inhibitor $p21^{cip1/waf1}$. We further explored the mechanism of $p21^{waf1/cip1}$ up-regulation in medulloblastoma cells. Perifosine did not increase p53 protein expression level, one of the main regulators of $p21^{waf1/cip1}$ transcription. In addition to p53, we also examined the effect of perifosine on other regulators of $p21^{waf1/cip1}$ including Akt pathway and MAPK pathway. Perifosine activates the MAPK (mitogen-activated protein kinase) signaling pathway in VC-312 without much effect in DAOY cells. However, studies are needed to explore the mechanism of perifosine induced $p21^{waf1/cip1}$ in medulloblastoma.

These experiments indicate that perifosine, either alone or in combination with other chemotherapeutic drugs, might be an effective therapeutic agent for the treatment of medulloblastoma.

CHAPTER 2

The alkylphospholipid perifosine induces apoptosis and $p21^{cip1/waf1}$ mediated cell cycle arrest in medulloblastoma

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Abstract

Medulloblastoma (MB) is the most common malignant cancer of the central nervous system in children. Akt kinases are part of a survival pathway that has been found to be significantly elevated in medulloblastoma. This pathway is a point of convergence for many growth factors and controls cellular processes that are critical for tumor cell survival and proliferation. The alkyl-phospholipid perifosine (octadecyl-(1, 1-dimethyl-4-piperidylio) phosphate) is a small molecule inhibitor in clinical trials in peripheral cancers which acts as an inhibitor of Akt kinases. Medulloblastoma cell cultures were used to study the effects of perifosine response in preclinical studies *in vitro*. Perifosine treatment led to rapid induction of cell death in medulloblastoma cell lines, with pronounced suppression of phosphorylated Akt in a time and concentration dependent manner. LD_{50} concentrations were established using viability assays for perifosine, cisplatin and etoposide. LD_{50} treatment of medulloblastoma cells with perifosine led to cleavage of caspase 9, caspase 7, caspase 3 and PARP, whereas caspase 8 was not detectable. Combination single dose treatment regimens of perifosine with sub-lethal doses of etoposide or irradiation demonstrated a greater than additive effect in medulloblastoma cells. Lower perifosine conentrations induced cell cycle arrest at the G1 and G2 cell cycle checkpoints, accompanied by increased expression of the cell cycle inhibitor $p21^{cip1/waf1}$. Treatment with p21 siRNA prevented the perifosine induced cell cycle arrest. These findings indicate that perifosine, either alone or in combination with other chemotherapeutic drugs, might be an effective therapeutic agent for the treatment of medulloblastoma.

Introduction

Medulloblastoma (MB) is the most common and fatal brain tumor among children, accounting for 12–25% of all pediatric tumors of the central nervous system (CNS) [114]. Medulloblastomas are most commonly characterized by highly mitotic small round cells with a high nuclear to cytoplasmic ratio, and classified as primitive neuro-ectodermal tumors (PNETs). The current treatment includes surgery, chemotherapy and radiation therapy. Current clinical trials include high dose chemotherapy for high risk and recurrent medulloblastoma, but few targeted small molecule inhibitors specific to medulloblastoma have been described. Despite these therapies, five year survival is at best 60-70%, and moreover, these therapies often affect the developing CNS, causing memory, attention, motor function, language and visuospatial deficits [106]. There is a need for the development of novel agents that can improve therapeutic results and avoid these deleterious treatment sequelae. Several signaling molecules have been associated with medulloblastoma development, including Sonic Hedgehog (Shh) [103] and members of the WNT pathway [46]. Recently Akt kinases, which were originally discovered as homologues to the oncogene in the thymoma-associated acute transforming retrovirus Akt-8 [123], have been found to have elevated activation levels in these tumors. Furthermore, elevated active Akt levels have been shown to be associated with features of malignancy such as proliferation, survival, glucose metabolism and revascularization in other cancers [9, 144]. Three Akt isotypes have been identified, Akt1, Akt2 and Akt3 [136]. It is currently unknown which of these isotypes are predominantly expressed in medulloblastoma. In animal models, exogenous activation of the Akt1 kinase pathway significantly enhanced Sonic Hedgehog–induced medulloblastoma formation [105]indicating a pro-malignancy interaction of these two pathways.

Due to the importance of Akt signaling in cancer biology, several small inhibitory molecules of Akt pathway have been developed for clinical use in cancer therapy [18]. As Akt activation is mediated by active upstream receptor proteins such as growth factor and adhesion receptors, and is activated largely within lipid-dependent protein signaling complexes at cell memebranes, phospholipid analogue compounds known as alkylphospholipids have been developed to interfere with this process, including perifosine[octadecyl-(1, 1-dimethyl-4-piperidylio) phosphate].

Perifosine is a novel phospholipid analogue which is currently undergoing phase I and phase II clinical evaluation [24, 74]. Although the exact mechanisms of action of perifosine are still being investigated, it is thought to interfere with the turnover and synthesis of endogenous membrane phospholipids, thereby affecting lipid- mediated signal transduction pathways, including inhibition of Akt [96], mitogen- activated protein kinase activation [111] and activation of c-Jun-NH_2-kinase (JNK). Perifosine is an orally bioavailable drug that has shown anti-tumor activity in preclinical models [69, 138]. Previously perifosine has been shown to induce apoptosis and cell cycle arrest in cancer cell lines [39, 77, 99]. However, perifosine has not been studied in medulloblastoma.

In this study our data demonstrate that endogenous active Akt (pAkt) is present at high levels compared to normal brain samples in medulloblastoma and derivative cell lines. Treatment of these cell lines with perifosine decreases active Akt levels in a dose- and time-dependent manner. We demonstrated that perifosine treatment led to rapid decreases in cell survival in medulloblastoma cells. In an attempt to understand the mechanism of perifosine mediated cytotoxicity, we examined the effect of perifosine on apoptotic regulatory proteins and

cell cycle distribution after treatment. Our data show that perifosine treatment led to up-regulation of caspase activity and programmed cell death mechanisms that are consistent with the intrinsic apoptotic pathway, including cleavage of caspase 9, caspase 7, caspase 3 and PARP in both cell lines and $p21^{waf1/cip1}$ -mediated cell cycle arrest. We also report that exposure to etoposide and radiation followed by a post-treatment with perifosine resulted in greater than additive effects on cell death, indicating that perifosine has chemo- and radio-sensitizing effects in medulloblastoma cells. These findings indicate that perifosine, either alone or in combination with other chemotherapeutic drugs, might be an effective therapeutic agent for the treatment of medulloblastoma, the most common malignant brain cancer in children.

Materials and Methods

Cell culture

Two medulloblastoma cell lines were used in this study, DAOY (from American Type Culture Collection), and VC-312, established under approved research protocols, and characterized in our laboratory (Pediatric Neuro-Oncology Laboratory, Virginia Commonwealth University). Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, glutamine and 1% penicillin-streptomycin solution at 37°C temperature with 5% CO2 in a humidified incubator.

Antibodies and Reagents

Antibodies against pan-Akt, phosphorylated Akt (Ser473 and Thr308), caspases-3,-7 and -9, and Erk1/2 were purchased from Cell Signaling Technologies (Beverly, MA). Anti-PARP antibody was obtained from Roche Applied Science (Indianapolis, IN). Anti- $p21^{waf1/cip1}$

monoclonal antibody was obtained from DAKO Cytomation (Carpenteria, CA). Mouse monoclonal P53 antibody and Phospho-Erk1/2 monoclonal antibody were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Perifosine was obtained from Keryx Pharmaceuticals (New York, NY) and was reconstituted as a 10 mM stock solution in sterile PBS. Etoposide and Cisplatin were purchased from Sigma-Aldrich (St. Louis, MO) and reconstituted in DMSO as stock solutions immediately prior to use.

Cell Lysis and Immunoblot Analysis

Cell preparations were subjected to lysis and protein extraction using RIPA lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5%SDS, 1%deoxycholic acid) containing protease and phosphatase inhibitors (EMD Biosciences; San Diego CA). Cell lysates were collected on ice and centrifuged for 15 min at 14,000 rpm, after shearing with a 1 ml syringe, fitted with 26 gauge needle. Supernatants were stored at -80^{0}C. Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by loading 20 ug or 40 ug of protein on Novex NuPAGE 4-12% Bis-Tris gels (Invitrogen), followed by electrophoresis for 55 minutes, and transferred to nitrocellulose membranes at 35 V for 2 hours (Invitrogen). After the transfer was completed, the protein blots were blocked in a buffer solution containing 5% non-fat milk or 5% purified bovine serum albumin (BSA) for 1 h at room temperature. BSA and non-fat milk are included to prevent non specific binding of antibody to the membrane due to low affinity non-specific interactions with non-target proteins; milk protein may contain phosphatases and alter the phosphorylation state of proteins of interest. Therefore BSA protein is used when phosphorylated epitopes are being examined with phosphorylation state-specific antibodies. The membranes were incubated with

primary antibodies overnight at 4°C and then washed four times in Tris-buffered saline containing 0.5% Tween-20 (TBST). After washing, the membranes were probed with anti-rabbit or anti-mouse secondary antibody (1:3,000-1:6,000, Rockland Inc., Rockland, ME, USA) conjugated with horseradish peroxidase for 1.5 h at room temperature. Western Blots were developed using the ECL Detection System (GE Healthcare-Amersham Biosciences, Piscataway, NJ, USA). β-Actin antibody (1:5,000, Sigma Biotechnology) was used as a control for protein loading.

Cell Viability Assays

Cell viability was determined using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI). Medulloblastoma cells were plated in white, opaque-walled, sterile, 96-well plates at the density of 1000 cells per 100 µL of growth medium per well. Cells were allowed to settle overnight. The next day cells were treated with the Akt inhibitor perifosine (1 µM to 50 µM), versus vehicle control for 24 to 72 hours. Viable cells were determined by adding Cell-titer Glo Luminescent Viability Assay lysis reagent (Promega, Madison, WI), incubating at room temperature with manual agitation for 2 minutes then on a rotating platform at 4°C for 10 min, and allowing equilibration for 15 minutes at room temperature. Luminescence was detected using a luminescent plate reader (Fluostar Optima, BMG Lab Technologies GMBH, Durham, NC). Mean relative light units for replicates within each condition were compared using Students t tests with the significance threshold set at 95% confidence ($p < 0.05$).

Exposure of cells to Ionizing Radiation

Cells (1 X 10^6) were plated in 100 mm dishes in complete media (5% FBS) and left overnight. The next day cells were irradiated with ^{60}Co γ rays at a dose rate of 1.1 Gy/min. After 24 hrs, cells were treated with different concentrations of perifosine. Cell viability was determined after 72 hrs in cell viability assays as described above.

Cell Cycle Analysis by Flow Cytometry

Cells (1 X 10^6) were plated in 100 mm dishes with growth media without serum. These cells were serum starved overnight for synchronization. Next day serum deficient media was replaced with complete media (5% FBS). Cells were treated with different concentrations of perifosine, and collected after a period of 12 or 24 hours. After treatment, cells were trypsinized and cell suspensions washed twice in PBS. Cell fixation was performed in 1 ml of 70% ethanol. After 30 minutes, cells were centrifuged and resuspended in 400 µl of propidium iodide/RNAse B solution (Apo-Direct Staining solution, BD Biosciences-Pharmigen; San Diego, CA) for 30 min at room temperature. Cells were measured on a FACScanto flow cytometer and data analyzed using FACSDiva 5.0 software (BD Biosciences).

Short interfering RNA transfection

Cells were transfected with $p21^{waf1/cip1}$ siRNA pre-designed validated oligonucleotides, containing sequences directed against $p21^{waf1/cip1}$ (ABI-Ambion). Cells were plated in six well plates in triplicate at the density of 2 X 10^5 cells per well, and allowed to attach overnight. The next day media was replaced with 500 µl Optimem containing 2% serum. Cells were transfected with siRNA to a final concentration of 25 nM. In 100 µl serum-free Optimem, 5µl of a 10 µM siRNA stock solution (Non-target control siRNA, Dharmacon, or p21WAF1 siRNA, Ambion)

was premixed with 3µl of oligofectamine for each respective well. Reagents were mixed well and incubated at room temperature for 2 minutes to allow complexes to form. After reagents for transfection were added to the respective wells and cells were placed on a platform rocker for 4 hours in a sterile incubator at 37^0 C. After 4 hours, cells were supplemented with 1500 µl of Optimem (10% serum), were taken off the rocker, and incubated for 20 additional hours. Twenty four hours after beginning the transfection, cells were treated with 15 µM perifosine for 24 hrs. Cells were collected for western blot analysis or fixed in 70% ethanol for flow cytometry studies.

Statistical Analysis

Data were evaluated by comparing the means and standard errors of the mean of replicate experiments. Data are expressed as the mean and standard error of the mean (SEM) of at least three independent experiments. Statistical analysis was performed using an unpaired Student's t-test. A p value <0.05 was considered significant.

Results

AKT isotype expression and its activation level in medulloblastoma.

To characterize endogenous protein expression levels of Akt isotypes, Akt1, Akt2, Akt3, and the phosphorylated forms of Akt at the two major phosphorylation sites, Threonine 308 and Serine 473, were examined by western blot under normal growth conditions. As shown in Figure 2.1A, Akt1 and Akt3 were detected in both cell lines at significant levels, whereas Akt2 protein was detected more predominantly in DAOY cells. Despite apparent differences in isotype expression levels, robust phosphorylated-Akt was detected in both cell lines using

antibodies non-selective for individual isotypes. These results suggest that even though the protein expression levels of Akt isoforms may vary in these cell lines, both have highly active endogenous Akt signaling. Transcript expression levels for each Akt isotype were also examined in medulloblastoma clinical specimens (n=11) compared with normal cerebellum (n=4). The abundance of messenger RNA was examined by Taqman QPCR assay. As shown in Figure 2.1B, mRNA levels for Akt1 were similar to normal brain, while Akt2 and Akt3 were significantly elevated in medulloblastoma, measured as a ratio to the internal control mRNA for β-actin.

Figure 2.1 Expression of Akt isoforms in medulloblastomas. A. Detection of all three Akt isoforms expressed in medulloblastoma cell lines. Western blotting analysis of protein lysates derived from DAOY and VC-312 were probed with isoform-specific antibodies for Akt1, Akt2, Akt3 and P-Akt-phospho-Serine 473. ß-actin is used as an internal control. B. Taqman assay of Akt isoforms in medulloblastoma tissues *in vivo* compared with normal cerebellum (n= 4 normal, black bars; n= 11 medulloblastoma, grey bars, *p< 0.05). The mRNA levels for Akt1 were similar to normal brain, while Akt2 and Akt3 were significantly elevated in medulloblastoma, measured as a ratio to the internal control mRNA for β-actin.

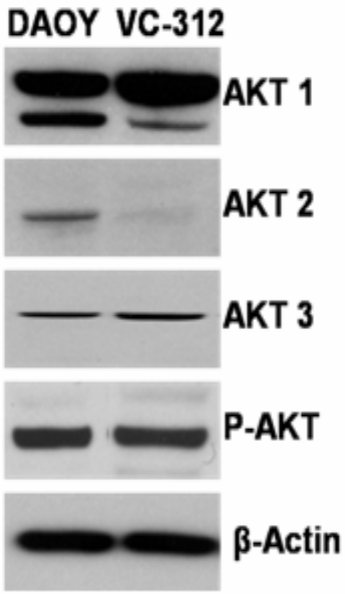


Figure 2.1 A

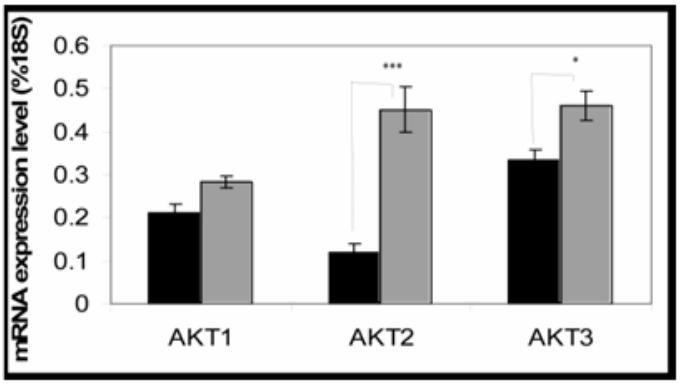


Figure 2.1 B

Suppression of Akt by perifosine in medulloblastoma

Perifosine impairs Akt phosphorylation by interfering with the binding of the PH domain of Akt to PIP3 [99]. We first examined the effect of perifosine on the phosphorylation status of Akt in medulloblastoma cell lines (Figure 2.2). Western blot analysis with phospho-specific AKT antibodies showed a decrease in phospho-Akt in a concentration- dependent manner at 3 hours. To determine the time-dependent effect of perifosine on phosphorylated active Akt levels, we treated DAOY and VC312 cells with 25 μM of perifosine (the approximate LD_{50} for both cell lines). There is complete loss of detectable phospho-Akt in both cell lines at this concentration by 6 hours. As Akt activity depends on its phosphorylation status and perifosine treatment leads to loss of phosphorylation, this indicates that perifosine induces inactivation of Akt in medulloblastoma cell lines.

Figure 2.2 Dose dependent suppression of active Akt by perifosine in DAOY and VC-312 cells. DAOY and VC-312 cells were treated with increasing doses (25-100 μM) of perifosine for 3 hours and cell lysates were prepared. Western blots of protein lysates were probed with antibodies to Akt and P-Akt-phospho-Serine 473. ß-actin is used as an internal loading control. The treatment groups were compared with control cells (C) which were treated with vehicle control. There is a decrease in phospho-Akt in a concentration- dependent manner at 3 hours.

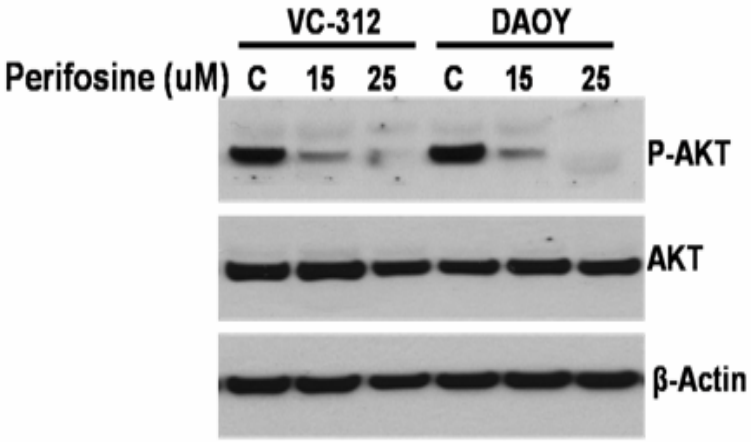


Figure 2.2

Inhibition of Akt decreases cell viability in medulloblastoma.

To determine whether perifosine treatment would result in a decrease in viability of medulloblastoma cells, DAOY and VC-312 cells were incubated in the presence of increasing concentrations of perifosine for 24 hrs. Cell viability was evaluated by Cell Titer-Glo luminescent ATP assay. Perifosine induced a dose dependent decrease in cell viability in both cell lines, shown in Figure 2.3. The LD_{50} (lethal dose to 50%) for DAOY and VC-312, determined using 3 replicate viability assays, was 25 µM. Rapid loss in viability was apparent at concentrations greater than 10 µM, and near-complete loss in survival was observed at 50 µM.

Figure 2.3 Effect of perifosine administration on medulloblastoma cell viability. Cells were seeded at a density of 10^3 cells per well in 96 well plate in replicates of six, and incubated for 24 hrs with 1, 10, 25, 50, and 100 µM perifosine. Relative cell number was measured by luminescent ATP viability assay. The cell viability (% Survival) was calculated with the following equation: % Viable cells (% Survival) = (x/y)*100 where 'x' is the number of viable cells in conditions exposed to perifosine, 'y' is number of cells treated with vehicle control. Error bars indicate standard deviation within an experiment, with significant differences determined using Student's T-test, $p < 0.05$. Representative experiments are shown from four independent trials.

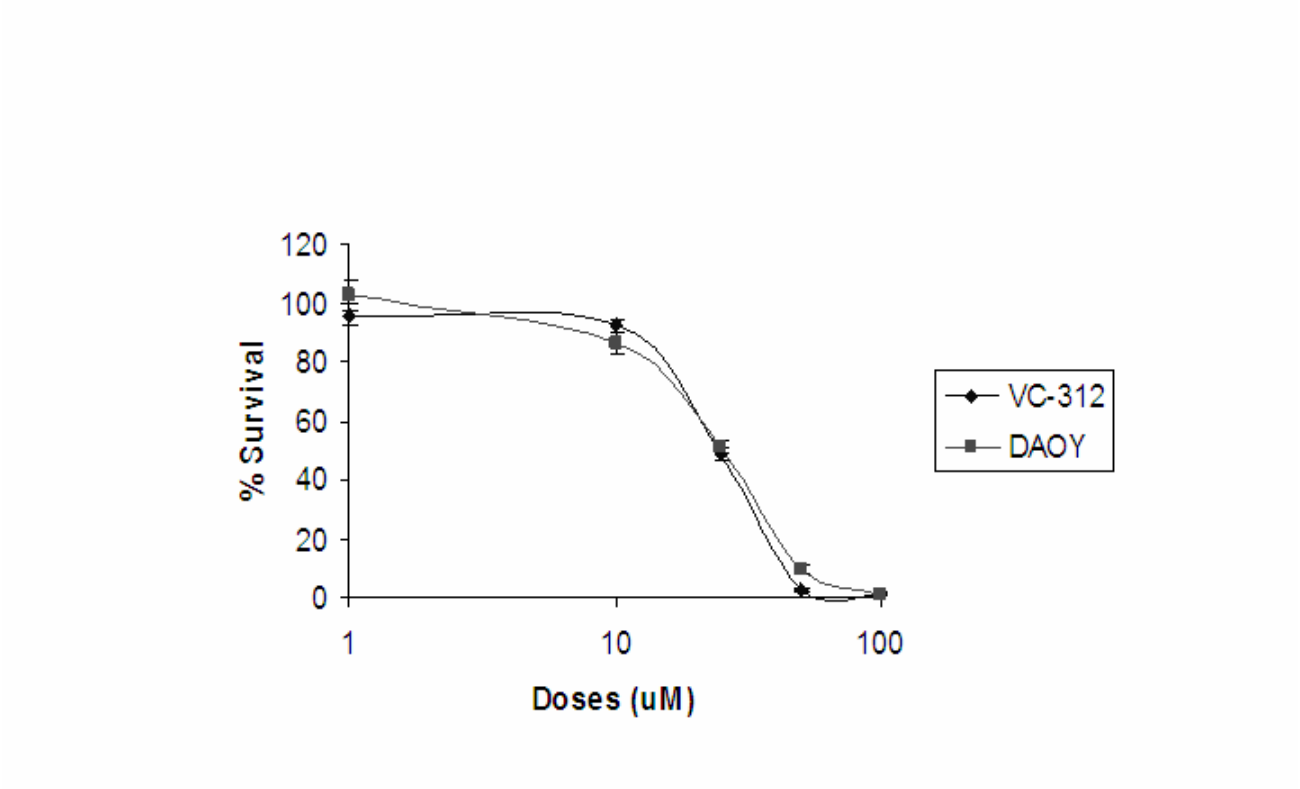


Figure 2.3

Effect of perifosine on apoptotic pathway

We further examined the induction of caspase cleavage in time course studies using concentrations at or above the established LD_{50} for perifosine. To determine the mechanism of cell death after perifosine treatment, medulloblastoma cells were treated with perifosine and examined for decreases in cellular proteins related to apoptosis. Poly-ADP ribosylation protein (PARP) and caspase cleavage have been used as sensitive indicators of cellular apoptosis. Cleavage of the effector caspases-3 and -7 and the upstream initiator caspase-9 was assayed by western blot using antibodies which detect pro-caspases and cleavage-specific forms indicative of activity. Treatment of DAOY and VC-312 cells with 25μM and 30 μM doses of perifosine, respectively, resulted in a time-dependent cleavage of caspases-9 (35 kDa), -3 (17/19 kDa), -7 (20 kDa) and PARP (85 kDa), shown in Figure 2.4. We were unable to detect caspase-8 in either cell line. As shown in Figure 2.4A and 2.4B, caspase-3 and PARP cleavage are detected by 6 hours in both cell lines, concurrent with loss of detectable phospho-Akt (Figure 2.4A). No significant change in total Akt is observed under the same conditions. Caspases-9 and 7 were also present in cleaved forms by 6 hours (total and cleaved forms shown for DAOY in Figure 2.4C).

Figure 2.4 Time dependent effect of perifosine on apoptotic induction. A. Effect of perifosine on phosphorylated Akt, total Akt, and caspase-3 cleavage, in DAOY and VC-312 cells. B. PARP cleavage detected in DAOY and VC-312 after perifosine. C. The effect of perifosine on caspase-9, and -7 cleavage in DAOY and VC-312 cells. DAOY and VC-312 cells were treated with 25 μM and 30 μM perifosine, respectively, and cell lysates were then subjected to western blotting. Treatment of DAOY and VC-312 cells with 25μM and 30 μM doses of perifosine, respectively, resulted in a time-dependent cleavage of caspases-9, -3, -7 and PARP.

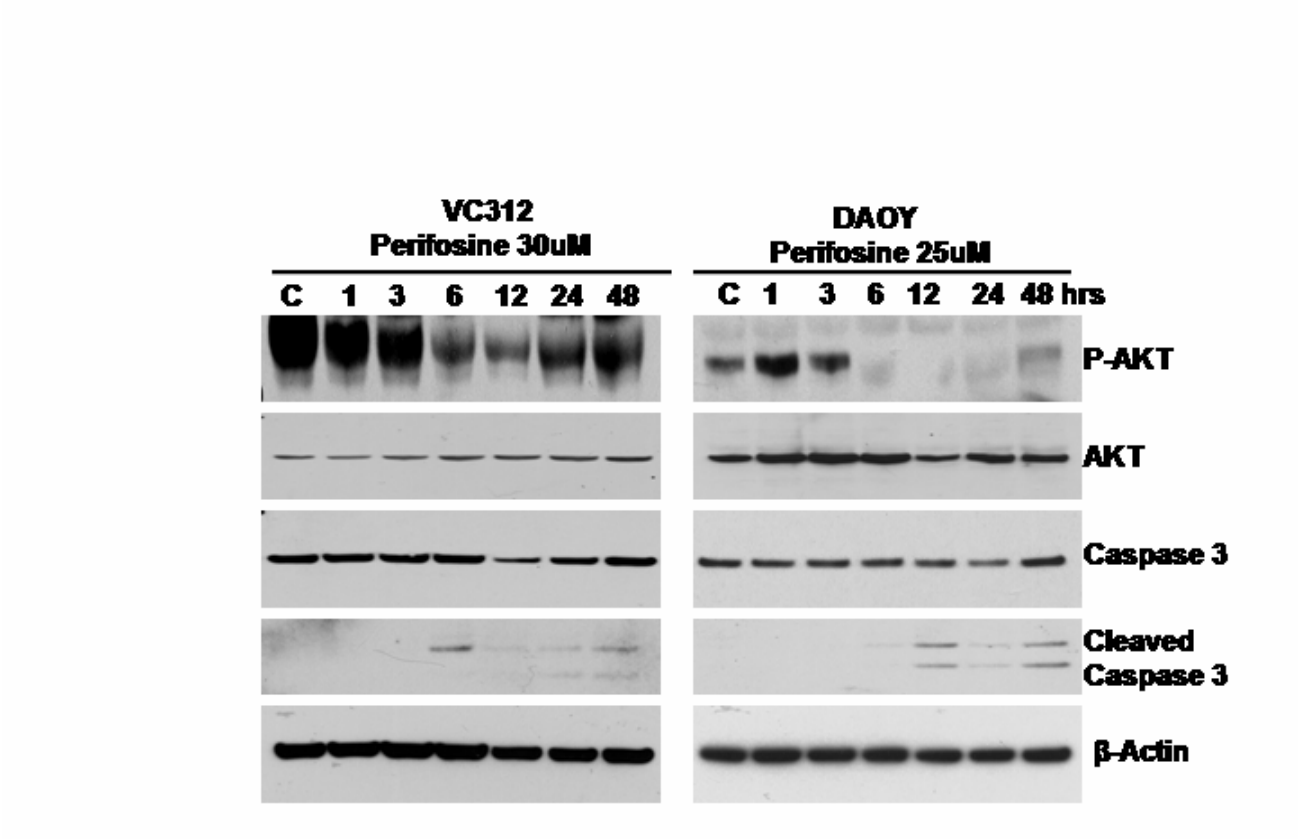


Figure 2.4 A

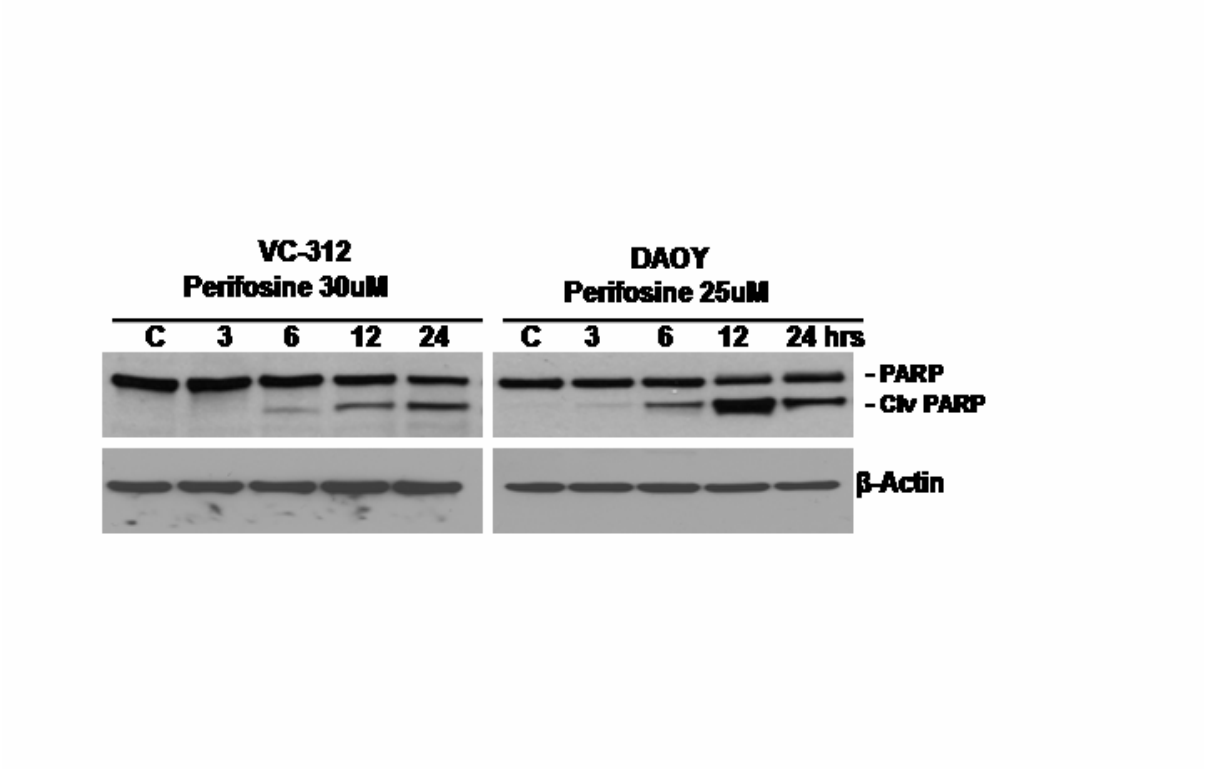


Figure 2.4 B

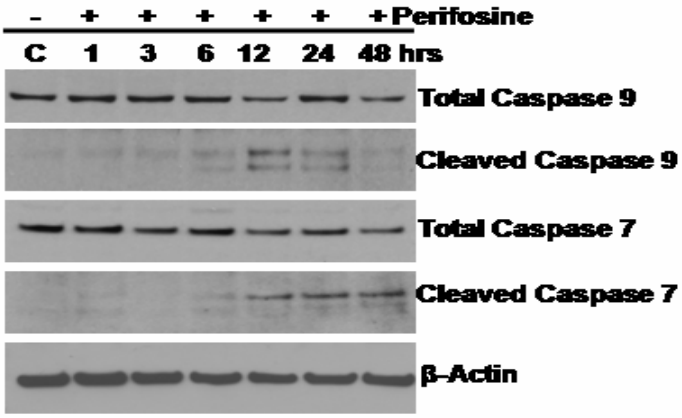


Figure 2.4 C

Perifosine sensitizes medulloblastoma cells to etoposide and radiation induced cytotoxicity

We next examined whether inhibition of the Akt survival pathway enhances etoposide and radiation induced cytotoxicity. The combined effect of perifosine with commonly administered chemotherapy drugs etoposide and cisplatin, or with ionizing radiation was evaluated in dose response studies. Figure 2.5 shows the dose dependent effects of etoposide in DAOY and VC312 cell. LD_{20} doses were first chosen to evaluate the combined effects of perifosine on cell viablilty. The effects of perifosine were additive in both cell lines. In contrast, low doses of etoposide with clinically achievable concentrations of perifosine (10 μM) generated greater than additive losses in cell survival in DAOY cells (Figure 2.5A). Comparison of mean cell survival values demonstrated significant differences between etoposide treatment alone and in combination with perifosine ($p= 0.0002$ and 0.0000007 for 0.1 μM etoposide and in combination with 10 or 20 uM perifosine) and versus VC312 and DAOY respectively). Dose-dependent cell survival studies with single dose ionizing radiation demonstrated LD_{50} values of 10 Gy for DAOY cells and for VC312 cells. LD_{10} values were used in combination treatments with increasing concentrations of perifosine (10-30 μM). As shown in Figure 2.5B, 8 Gy treatment of DAOY cells and 10 uM perifosine caused a significant increase in cell killing compared to either treatment alone ($p = 0.0032$ and 0.0013 for combination treatment with 8 Gy single dose and 10 and 20 μM perifosine, respectively). Combination index (CI) values were used to describe combined drug effect. Synergistic effect is considered when $CI < 0.85$ and antagonistic when $CI > 1.1$ and additive when values are close to 1. CI values calculated under optimized conditions for DAOY were determined to be 0.84126 for DAOY treated 72 hours with 0.1 μM etoposide and 10 μM perifosine, and 0.783 for VC312 cells under the same conditions. Similarly, after 8 Gy irradiations, the CI values for combined treatment with 10 μM were 0.73

for DAOY and 1.1 for VC312, indicating synergy in DAOY, but not in VC312 at the doses examined.

Figure 2.5 Perifosine co-treatment augments cell death by etoposide and irradiation in medulloblastoma cells. DAOY cells were exposed for 72 hrs to the indicated concentration of etoposide (0.1 µM) (A) and radiation 8 Gy (8) alone or in combination with 10, 15 and 20 µM perifosine added after 24 hrs. Cell viability was evaluated by ATP viability assay. The cell viability (% Survival) was calculated with the following equation: % Viable cells (% Survival) = (x/y)*100 where 'x' is the number of viable cells in conditions exposed to perifosine, 'y' is number of cells treated with vehicle control. Combination effect was determined by combination index (CI).

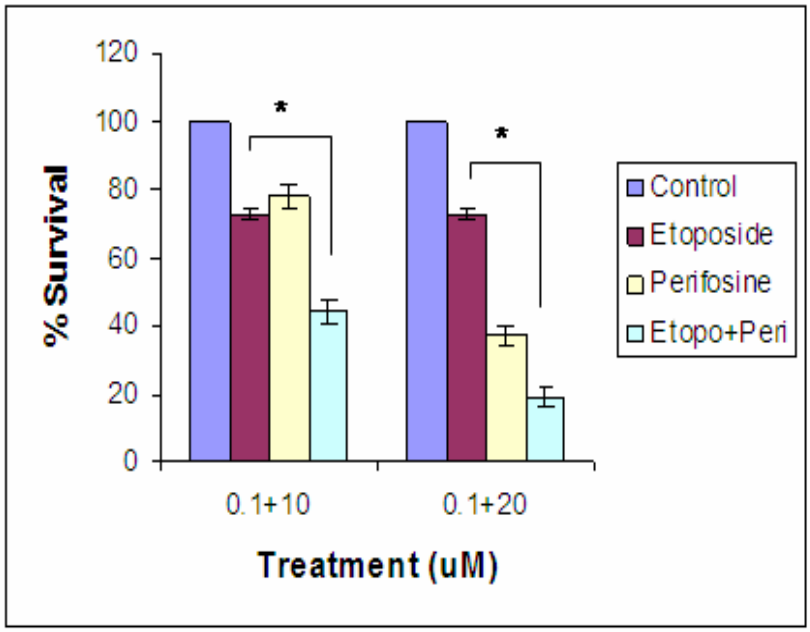


Figure 2.5 A

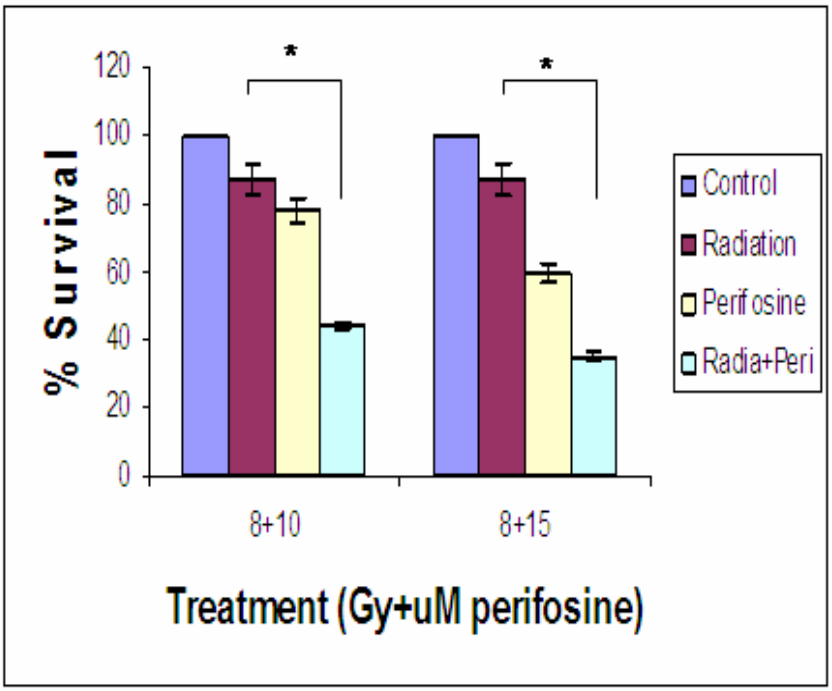


Figure 2.5 B

Effect of perifosine on cell cycle in medulloblastoma cells

To examine mechanisms other than caspase-mediated apoptosis responsible for the cytotoxic effects of perifosine, and because of the reported link of Akt activity with the cell cycle in medulloblastoma and other cancers, we sought to investigate the effect of perifosine on DAOY and VC-312 cell proliferation. To study dose dependent effects, DAOY and VC-312 cells were exposed to increasing concentrations of perifosine (5-30 μM) for 12 and 24 hours and then analyzed for cell cycle profiles by determining the DNA content of treated cell populations (Figure 2.6). Minimal effects were seen on cell cycle at the 5 μM dose of perifosine in DAOY and VC-312. The maximum effect was seen at 15 μM dose of perifosine, in which there was a significant increase in the G2-M phase population (paired T-test, $p= 0.00039$) and decrease in G0/G1 and S phase ($p= 0.00038$ and 0.017, respectively) of DNA in VC-312 cells (Figure 2.6B). In contrast to VC-312, perifosine treatment led to a significant increase in G0/G1 ($p=0.00117$) and decrease in G2-M phase ($p=0.0134$) in DAOY cells (Figure 2.6C). Further examination of the time-dependency of the observed growth arrest determined that there was a significant difference at 24 hrs as compared to 12 hours, consistent with an accumulation of treated cells at the cell cycle checkpoint.

Figure 2.6 Perifosine arrests proliferating medulloblastoma cells at cell cycle checkpoints. Exponentially growing DAOY and VC-312 cells were treated with perifosine (15μM), and analyzed by flow cytometry. A. Representative histograms obtained from flow cytometric analysis of cellular DNA content after staining with propidium iodide (PI). B. Analysis of mean % total cells from three independent trials. Means compared with T-test. Error bars indicate standard error of the mean, with significance set at p=0.05. Inset values: Relative % cells in Go/G1, S, and G2/M. DNA content was analyzed in medulloblastoma cells treated with 15 μM perifosine for 24 hrs. Perifosine treatment increases the G2-M phase population and decreases G0/G1 and S phase populations in VC-312 cells. In contrast to VC-312, perifosine treatment of DAOY cells led to a significant increase in G0/G1 and decrease in G2-M phase.

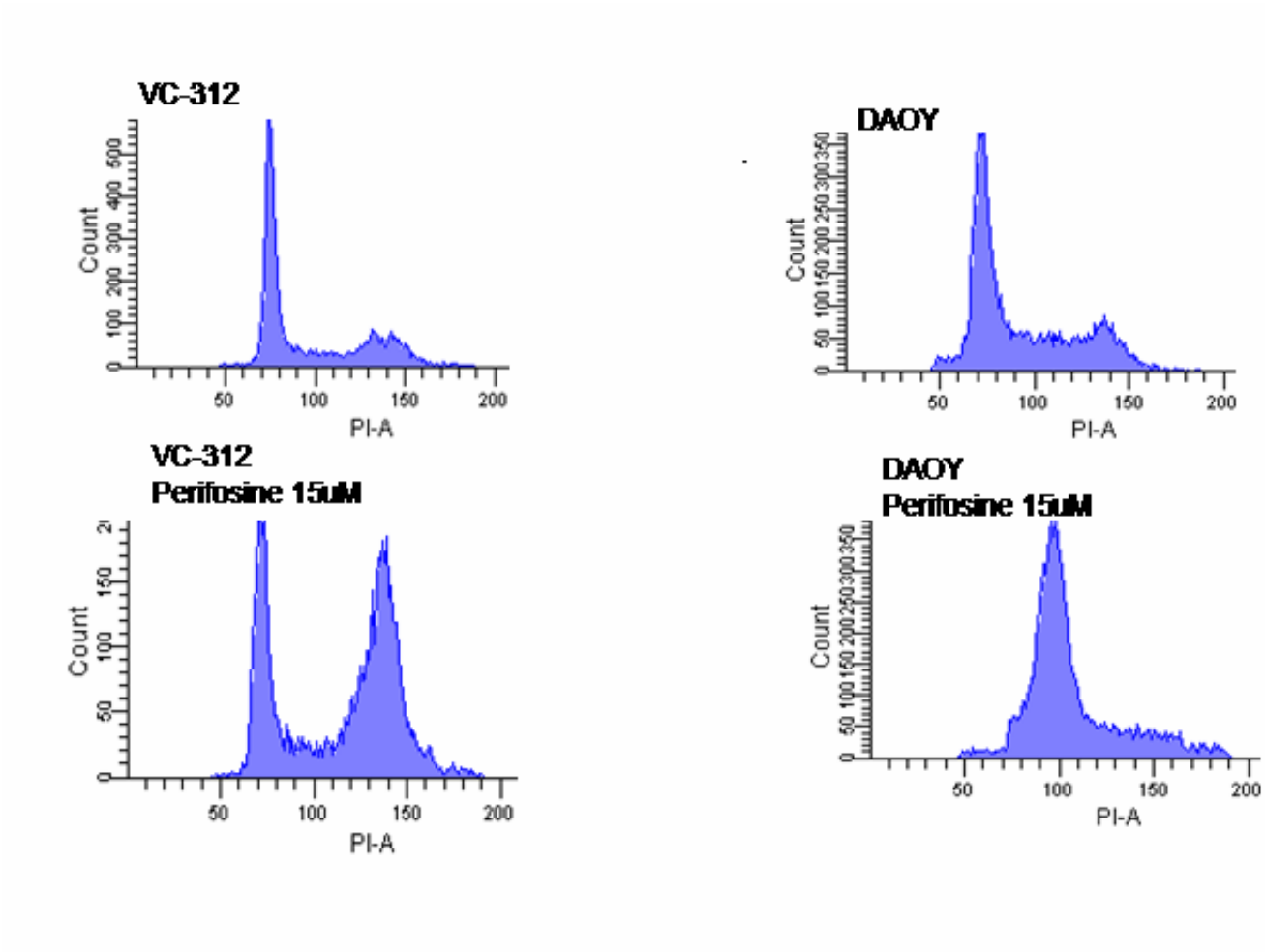
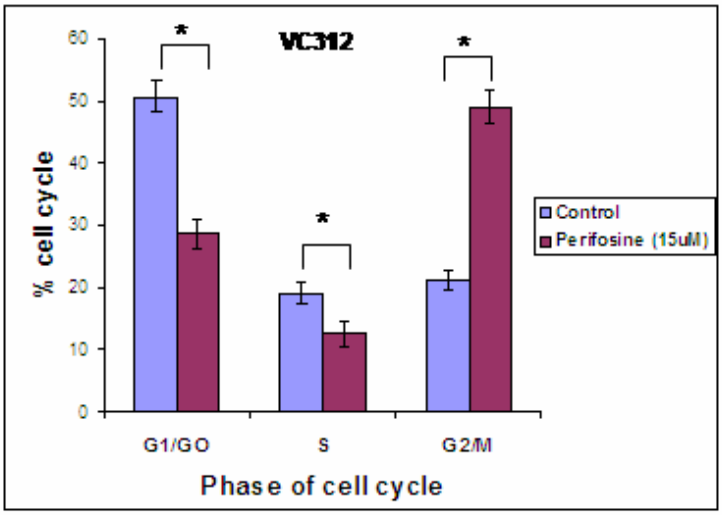


Figure 2.6 A



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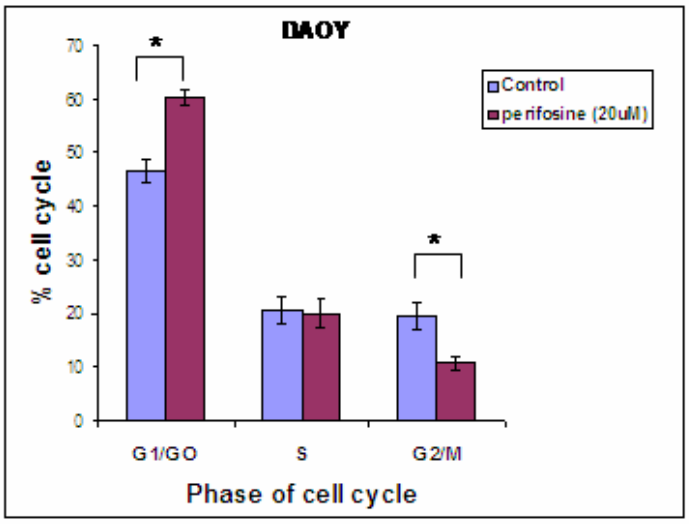

Figure 2.6 C

Dependence of perifosine induced cell cycle arrest on p21waf1/cip1

To determine which cell cycle regulatory proteins were involved in the observed cell cycle arrest following perifosine treatment, western blot analysis of control and treated cell lysates was performed. The $p21^{waf1/cip1}$, CDK inhibitor protein level was found to be robustly increased by perifosine in a dose-dependent manner, within 6 hours of treatment. As shown in figure 2.7, there is a marked increase in $p21^{waf1/cip1}$ protein expression in DAOY at 15 µM (mean 5.28, SD 0.21) and 25 µM (mean 4.82 fold, SD 0.45). Similarly an increase was found in VC-312 cells at 15 µM (mean 5.08 fold, SD 0.51) and 25µM (mean 10.4, SD 1.42). To further investigate the role of $p21^{waf1/cip1}$ in perifosine induced cell cycle arrest, small interfering RNA duplexes were transfected into the two cell lines to knock down $p21^{waf1/cip1}$ protein expression prior to perifosine treatment. After successful knock down of $p21^{waf1/cip1}$, shown by western blotting in Figure 2.8A, flow cytometric analyses were repeated to analyze cell cycle distribution. Loss of $p21^{waf1/cip1}$ by RNA interference prevented the perifosine induced cell cycle arrest, whereas perifosine induced arrest was again demonstrated at cell cycle checkpoints in non- transfected controls and non-targeting siRNA control cell populations (Figure 2.8B).

Figure 2.7 Perifosine induced cell cycle arrest is associated with induction of $p21^{waf1/cip1}$. DAOY and VC-312 cells were exposed with perifosine (15 μM) for 12 hrs. Cell lysates were prepared and western blotting was performed. The membrane was probed with $p21^{waf1/cip1}$. β-Actin was used to estimate equal protein loading. $P21^{waf1/cip1}$ expression increased on exposure to perifosine in both cell lines.

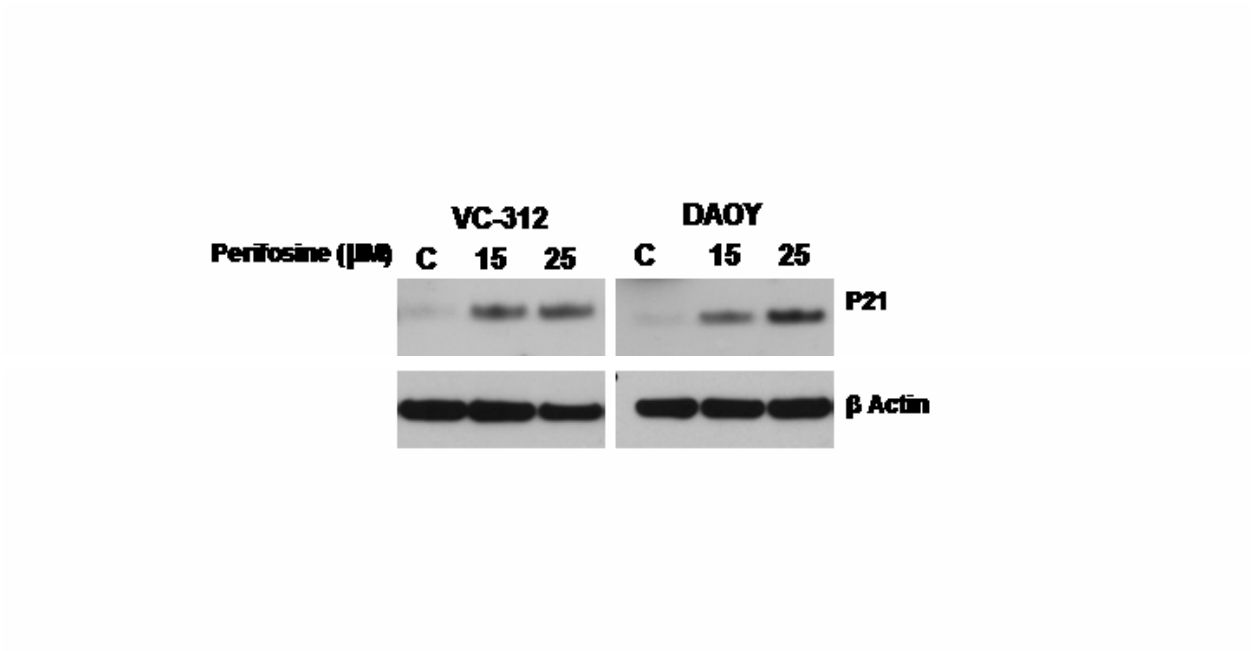

Figure 2.7

Figure 2.8 Suppression of $p21^{waf1/cip1}$ by RNA interference reverses perifosine-induced cell cycle arrest in medulloblastoma. Cells were plated in six well plates in triplicate at the density of 2 X 10^5 cells per well, and allowed to attach overnight. Transfection was carried out with siRNA using oligofectamine and Optimem as described in the text. Twenty four hours after transfection, cells were treated with 15 μM of perifosine for 24 hrs. Cells were collected for western blotting analysis or fixed in 70% ethanol for flow cytometry studies. A. Suppression of $p21^{waf1/cip1}$ protein expression is observed by western blotting 48 hours after transfection. B. Cell cycle analysis of $p21^{waf1/cip1}$ siRNA and control treated medulloblastoma cells in the presence or absence of perifosine (15 μM).

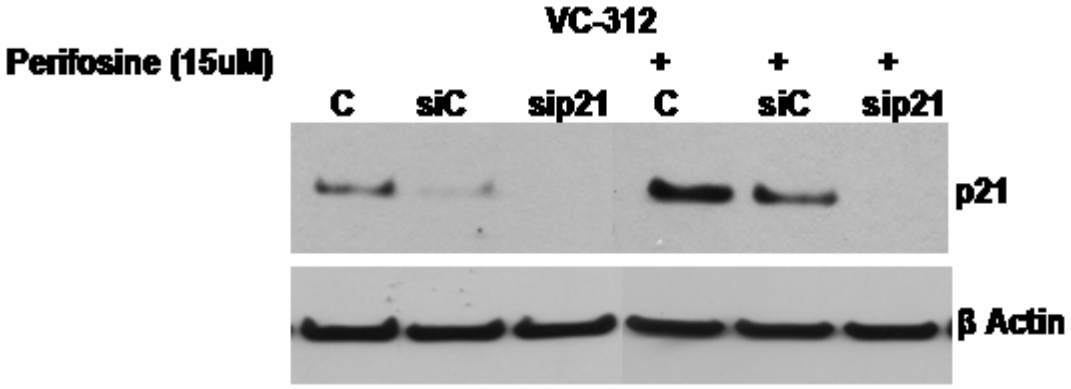

Figure 2.8 A

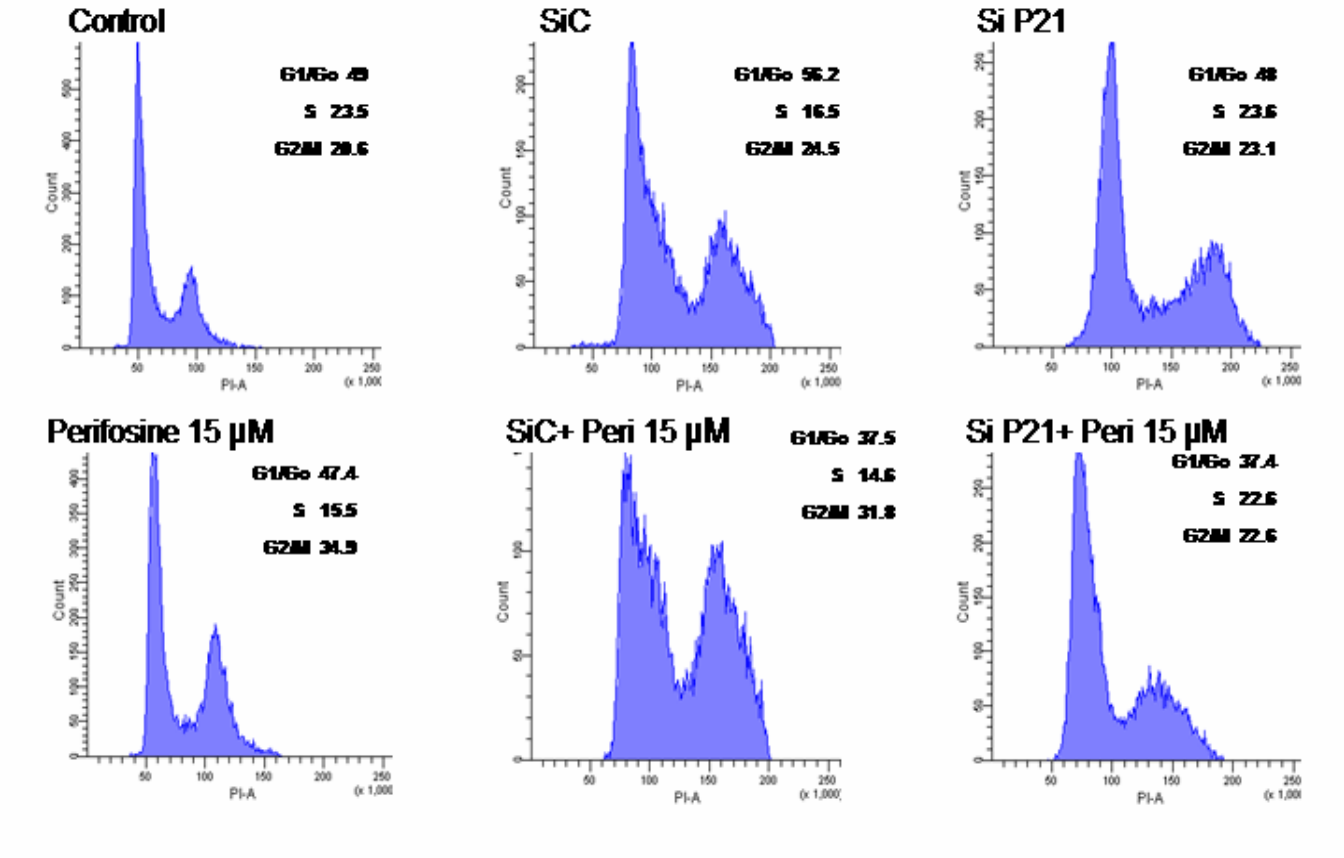

Figure 2.8 B

Discussion

Medulloblastomas are the most common malignant pediatric brain tumors, but their molecular pathology is not fully understood. The phosphatidylinositol 3'-kinase -mediated Akt signaling pathway has been found to have a role in tumor cell survival and proliferation (20). Recently, elevated activation of the PI3K/Akt signaling pathway has been found to be a common event in medulloblastomas [60]. The activation of Akt signaling has been attributed to deregulation of different components of the PI3K/Akt pathway, including PTEN deletion [124], PI3K gene amplification, Akt amplification [110], as well as Akt over expression [140]. The exact mechanism of Akt activation in medulloblastomas is still unknown, but one possible mechanism is reduced expression of PTEN [60]. Perifosine (octadecyl-[*N*,*N*-dimethyl-piperidinio-4-yl]-phosphate), a synthetic alkyl-lysophospholipid structurally related to ether lipids, is thought to interfere with Akt mediated signal transduction pathways after it is internalized via raft-mediated endocytosis [134]. Here we have shown the efficacy of perifosine in decreasing phosphorylated Akt in DAOY and VC-312 medulloblastoma cell lines. Perifosine, in a dose-dependent manner, decreased cell survival of both cell lines, and the loss of cell viability followed a marked reduction in phospho-Akt-S473. Perifosine treatment decreased phospho-Akt levels in both a dose- and time-dependent manner. This data is in accordance with previously reported data of perifosine action on other cell lines [95]. Perifosine treatment resulted in up-regulation of P-ERK in medulloblastoma cell lines. The up-regulation of Erk signaling could be because of activation of Raf-mediated activation of mitogen activated signaling pathway as P-AKT has been reported to negatively regulate Raf-1 [125]. The decreased cell survival in medulloblastoma was due to apoptosis and a decrease in cell cycle progression, as indicated by western blot and flow cytometric analysis of samples treated with perifosine. Perifosine treatment resulted in

activation of multiple caspases including initiator caspase-9, and effector caspase-3 and -7. Perifosine treatment also led to cleavage of PARP, which is a caspase-3 substrate. Inhibition of caspase-3 using pharmacological inhibitors attenuated the perifosine induced decrease in cell viability (Appendix B); substantiating the role of caspase activation in perifosine induced cell death. Perifosine treatment has been found to induce activation of the Fas/CD95 death receptor in multiple myeloma cells, leading to caspase-8 activation, in turn leading to cleavage of Bid, and subsequent caspase 9 activation [44, 55]. Perifosine might have similar mechanisms of action in medulloblastomas. However, we were unable to detect caspase-8 expression in either cell line examined, consistent with reports of epigenetic silencing of caspase-8 in medulloblastoma [51].

To examine the cytotoxic effect of perifosine in more detail, cell cycle progression of medulloblastoma cell lines exposed to perifosine was examined. We observed that a 15 uM perifosine treatment for 24 hrs led to accumulation of DAOY cells in G_1 phase and VC-312 cells in the G_2-M phase of cell cycle. As cell cycle progression is governed by the cyclical activation of cyclin dependent kinases (CDKs), which are regulated by cyclins and cdk inhibitors ($p21^{waf1/cip1}$, p27) [118], it was of interest to evaluate the effect of perifosine on these cell cycle regulatory proteins. Unlike most chemotherapeutic anticancer drugs, which target DNA, perifosine is inserted in the plasma membrane and is thought to interfere with signal transduction pathways that are critical for cell survival. Due to their distinct mode of action, alkylphospholipid drugs are considered as attractive candidates to combine with chemotherapy and radiotherapy to overcome therapeutic resistance [139]. In addition, there is limited efficacy of perifosine monotherapy reported in a variety of solid malignancies. We also tested the effect of perifosine on etoposide and radiation-induced cell death in the two human medulloblastomas cell lines. Perifosine enhanced etoposide and radiation-induced cell death. This effect was additive

for VC-312 and synergistic for DAOY, resulting in marked increase in cell death. Etoposide is a DNA damaging anticancer drug which targets DNA topoisomerases, interfering with DNA structural modification during DNA synthesis and mitosis, and thereby disabling mitotic progression. Topoisomerase inhibitors have been shown to have inhibitory actions on cell cycle progression in late S and G2/M phases of the cell cycle [128]. This inhibitory mechanism on S-phase progression results in late S-phase and G_2-M phase arrest, reflective of a DNA repair process in progress, and leading eventually to mitotic catastrophe and cell death [36]. In leukemia cells, in addition to G_2-M arrest, which results in mitotic cell death, a concurrent induction of apoptosis occurs [104]. The observed effects of perifosine on cell cycle arrest in medulloblastoma cells in the present study is similar to that observed in human T cell leukemia, which show a synergistic cell death effect when co-treated with etoposide [95]. The apparent synergy seen in the current study was observed at clinically achievable concentrations of perifosine, which is encouraging for further development of optimal dosing regimens in pediatric patients. Perifosine appears to be a promising adjuvant treatment, deserving of further evaluation in pediatric brain tumors such as medulloblastoma.

Acknowledgements

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CHAPTER 3

Effect of perifosine on cell cycle and p21$^{waf1/cip1}$ regulatory proteins in human medulloblastoma cells

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Abstract

Perifosine is a novel alkyl-lysophospholipid drugs which upon exposure to cellular membranes becomes inserted into the cell membrane and affects lipid synthesis and turnover. Perifosine has shown promising preclinical activity in many tumors, including leukemia and prostatic carcinoma cells. Similar to other cancer cell types, perifosine has strong antineoplastic potential against medulloblastoma cells. In the previous chapter we studied the effects of perifosine on apoptotic regulatory proteins in medulloblastoma but perifosine effects on cell cycle regulatory proteins are not well established. We therefore, assessed the effect of perifosine on the cell-cycle regulatory molecules Cyclin D1, phospho-Cdc2 as well as the involvement of Akt, Erk 1/2 and p53 proteins in two cell lines of medulloblastoma (VC-312 and DAOY). There was no change in Cyclin D1 expression in DAOY cells treated with perifosine, whereas VC-312 cells showed an increase in Cyclin D1 levels. Perifosine treatment did not significantly affect the levels of phospho-Cdc2 protein expression in either cell line. We also explored whether a decrease in pAkt or an increase in pErk in response to perifosine mediates induction of $p21^{waf1/cip1}$. Perifosine mediated induction of $p21^{waf1/cip1}$ levels in medulloblastoma cells could not be prevented by constitutive activation of Akt, nor by an increase in phosphorylation of Erk1/2. Furthermore, we determine the role p53 in perifosine induced $p21^{waf1/cip1}$. Our findings show that perifosine treatment does not increase the overall protein levels of p53. Further studies are required to determine the critical signaling pathways mediating perifosine mediated $p21^{waf1/cip1}$ response in medulloblastoma.

Introduction

Most chemotherapeutic drugs currently used for treating medulloblastoma act by targeting dividing cells and thus could also potentially affect normal brain and peripheral cells thus leading to significant side effects. An alternative or additional approach for treating medulloblastoma would be to use drugs that target specific tumor promoting proteins (onco-proteins) over-expressed by tumor these cells. The aberrant expression of these onco-proteins is believed to activate cell-signaling pathways including PI3K/Akt.[144]. These activated cell signaling pathways could thus be selectively targeted by drugs such as perifosine. Perifosine acts by interfering with phospholipid synthesis and turnover. We have shown that perifosine has strong antineoplastic activity against medulloblastoma cells that involves the induction of apoptosis and cell cycle arrest. In the last chapter we had shown an effect of perifosine on apoptotic regulatory proteins. However, the effect of perifosine on cell cycle regulatory proteins in medulloblastoma was not established.

There is a large body of literature which suggests that alterations of specific cell cycle regulatory proteins are frequent and necessary events underlying the development of tumors [117]. Thus, modulation of these proteins is an attractive avenue for the therapy and prevention of human neoplasms. The cell cycle regulatory proteins include cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors[132]. Activity of cdks is regulated by the cyclins (positive regulators) and the cyclin-dependent kinase inhibitors (CKIs, negative regulators). Medulloblastoma cells have been shown to have mutations in highly conserved beta-catenin phosphorylation sites involved in protein stability[34]. Increased beta-catenin has been shown to increase the expression of Cyclin D[76]. Absence of Cyclin D proteins drastically decreases the incidence of

medulloblastoma formation in murine models[101]. Loss of cdk inhibitor p18 (INK4C) protein expression was detected in a significant fraction of human medulloblastoma specimens, pointing toward a role for INK4C in suppression of medulloblastoma formation. These findings suggest the involvement of these proteins in the development of human meduloblastoma[88]. In this study, we determined the effect of perifosine on cell-cycle regulatory proteins including cyclins and cyclin dependent kinase signaling pathways.

In our studies perifosine treatment increased the expression of Cyclin D1 in VC-312 short-term medulloblastoma cultures whereas minimal changes were detected in DAOY cells. Perifosine treatment does not have a significant effect on phosphorylated-Cdc2 protein levels. We also showed that perifosine treatment decreased the expression of p53 (a transcriptional activator of $p21^{waf1/cip1}$) at early post-treatment time points (12 hours) in VC-312 cells, whereas the same treatment did not have significant effect on p53 levels in DAOY cells. Finally, we determined the involvement of Akt and MAPK signaling in perifosine-mediated increased in $p21^{waf1/cip1}$. Perifosine decreased phosphorylated Akt in both cell lines and increases phospho-ERK1/2 in VC-312 cells. These finding suggest that perifosine-mediated increase in $p21^{waf1/cip1}$ does not appear to require a decrease in phospho-Akt nor an increase in phosphorylated Erk1/2. Therefore, the mechanism of the perifosine mediated increase in $p21^{waf1/cip1}$, which is critical for perifosine induced cell cycle arrest in medulloblastoma cells, is still unclear.

Materials and Methods

Cell culture

Human medulloblastoma derived cell lines (DAOY, VC-312) were used in this study. DAOY cells line was obtained from American Type Culture Collection (ATCC), and VC-312

cell line was established and characterized in our laboratory (Pediatric Neuro-Oncology Laboratory, Virginia Commonwealth University). The primary culture of VC-312 was obtained under approved research protocol from a pediatric medulloblastoma patient who underwent surgery at the Virginia Commonwealth University Health System, Medical College of Virginia Hospital. The tumor tissue was dissected with a scalpel and dissociated in to small pieces separating it from connective tissue and blood vessels. The dissected tissue was washed twice in PBS and centrifuged at 300 x g, and the resulting pellet was resuspended in 3 ml of Dulbecco's PBS (DPBS) containing 200 units papain solution (Roche) and 2 units DNase I (Sigma-Aldrich). After trituration, the pellet was incubated for 30 minutes, with trituration every 10 minutes, followed by ten passes through a sterile 18 gauge syringe, one pass through a 35 µM mesh filter, and centrifugation. The resulting pellet wash washed twice in DPBS, centrifuging between each wash. The final pellet was evenly distributed into each well of a six-well tissue culture plate, and cultured in DMEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 1% N-2 supplement (Invitrogen), 20 ng/mL recombinant human epidermal growth factor (Becton Dickinson), and 10 ng/mL recombinant human basic fibroblast growth factor (Becton Dickinson)), 1% antibiotic-antimycotic solution (Invitrogen). VC312 cultures were incubated in a humidified incubator at 37°C with 5% CO_2 in 95% air. Primary and subsequent passages of VC312 were cryopreserved and all experiments were performed using cells initiated from passage 5 stock vials. After establishing the cell line, these cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, 1% penicillin-streptomycin solution at 37°C temperature with 5% CO2 in a humidified incubator. When cells reached 80% confluence, they were removed and suspended using a trypsin–EDTA

solution (0.05% trypsin), washed, centrifuged, and replated. The culture medium was changed every second day after plating.

Antibodies and Reagents

Antibodies against pan-AKT, phosphorylated AKT (Ser473), and Erk1/2 were purchased from Cell Signaling Technologies (Beverly, MA). Anti- $p21^{cip1/waf1}$ monoclonal antibody was obtained from DAKO Cytomation (Carpenteria, CA). Mouse monoclonal P53 antibody and Phospho-Erk1/2 monoclonal antibody were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Perifosine was obtained from Keryx Pharmaceuticals (New York, NY) and was reconstituted as a 10 mM stock solution in sterile PBS.

Protein Extraction

Protein was extracted from medulloblastoma cell lines using Radio-Immunoprecipitation Assay buffer (RIPA buffer). The ingredients of RIPA buffer include: 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. Protease and phosphatase inhibitors (EMD Biosciences; San Diego CA) are added prior to use (100uL of each in 10mL of RIPA). Cells were plated in six well plates (at a density of 2×10^6 cells per well) overnight. At the time of protein extraction media was removed and the cells were washed with ice cold PBS. The six well plates were transferred to ice and 200 μL of RIPA was added to each well. After 5 minutes cells were scraped and sheared with a 1 ml syringe, fitted with 26 gauge needle. Fifteen minutes after adding RIPA, cells were scraped and transferred to 1.5mL Eppendorf tubes. Cell lysates were then centrifuged at maximum speed in a table top centrifuge for 20 minutes at 4^o C. The supernatant was transferred to another fresh labeled Eppendorf tube. Protein

concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot

Protein samples were separated using denaturing polyacrylamide gel electrophoresis (PAGE). Cell lysates were taken from aliquots stored at -80^0 C and were thawed on ice. The NuPAGE® loading Buffer (10X) was mixed with proteins (20ug or 40ug) along with antioxidant and nanopure water protein and heated at 70°C for 10 minutes. The proteins were resolved on a Novex NuPAGE 4-12% Bis-Tris gels (Invitrogen), followed by electrophoresis for 55 minutes, and transferred to nitrocellulose membranes at 35 V for 2 hours (Invitrogen). After the transfer was completed, the protein blots were blocked in a buffer solution containing 5% non-fat milk or 5% BSA for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C and then washed four times in Tris-buffered saline containing 0.5% Tween-20 (TBST). After washing, the membranes were probed with anti-rabbit or anti-mouse secondary antibody (1:3,000-1:6,000, Rockland Inc., Rockland, ME, USA) conjugated with horseradish peroxidase for 1.5 h at room temperature. Western Blots were developed using the ECL Detection System (GE Healthcare-Amersham Biosciences, Piscataway, NJ, USA). β-Actin antibody (1:5,000, Sigma Biotechnology) was used as a control for protein loading.

Pharmacologic treatments

DAOY and VC-312 cells were plated in six well plates at the density of 2x10^5 cells per well in DMEM with 10% FBS. Cells were pretreated with U0126 (10 µM) for 1 hr and then perifosine was added (15 µM, 25 µM) for 12 hrs. Cell lysates were collected for immunoblotting.

Adenoviral transfection

Constructs encoding constitutively active AKT (CA-Akt) or dominant negative AKT (DN-Akt) in adenoviral expression plasmids were kindly provided by collaborator Dr. Paul Dent (Department of Biochemistry, VCU). Adv-CA-Akt; adenovirus encoding constitutively active Akt1 is a replication defective adenovirus encoding a plasmid constructed to express constitutive active Akt1. Active forms of Akt have been obtained by fusion of NH_2 -terminal c-Src myristoylation residues to Akt1, and its dominant negative form mutated by encoding alanine in place of the Serine473 and the Threonine308 position. This functionally blocks phosphorylation by upstream kinases, resulting in a dominant negative Akt. Cells were seeded in six well plates in triplicate at the density of 2 X 10^5 and allowed to attach overnight. The following day, cell growth media was replaced with 600 µl Optimem (antibiotic free with 2% serum; Invitrogen). Medulloblastoma cells were infected with adenovirus containing constitutively active AKT, or dominant negative AKT. The viral MOI (multiplicity of infection) used for infection ranged from 3 to 30. Control CMV-LacZ adenovirus was used as a control with the same MOI as the recombinant AKT virus. Cells were rocked for 4 hours at 37^0 C. After 4 hours, cells were supplemented with 1200 µl of Optimem (no antibiotics with 10% serum) for 20 hrs. Cells were incubated for a further 24 hours (48 hour's total) to ensure adequate expression of the transduced gene product. After transduction, cells were treated with 15 µM of perifosine for 24 hrs. Cell lysates were collected for western blot.

Results

Effect of perifosine on cyclins and cyclin dependent kinase (cdk) in medulloblastoma cells.

Since previous studies showed perifosine treatment results in accumulation of cells in G_2/M phase in VC-312 cells and G1/S phase in DAOY cells, we investigated whether

differential effects of perifosine on cyclins and cdk inhibitor proteins may explain this difference. In the previous chapter we reported the effect of perifosine on the cdk inhibitor $p21^{waf1/cip1}$. Here we determined the effect of perifosine on cyclins. Cells were treated with perifosine (15 µM and 25 µM) for 12 hrs and 24 hrs and protein expression of Cyclin B, Cyclin D1 was examined by western blot analysis. DAOY cells demonstrated no change ($p > 0.05$) in protein expression on exposure to perifosine (15 µM, 25 µM) for 12 hrs and no change at 24 hrs (Figure 3.1). VC-312 cells showed a statistically significant increase in Cyclin D1 protein levels on exposure to perifosine for 12 hrs (mean 1.82 fold, SD 0.08) and 24 hrs (mean 1.89 fold, SD 0.04) (Figure 3.1). Cyclin B protein expression was not detected in both cell lines, under the conditions used. Lastly, we determined the effect of perifosine on cyclin dependent kinase Cdc2 in these cells. Cells were treated with perifosine (15 µM, 25 µM) and protein expression of phosphorylated Cdc2 was determined. There was no significant change in phospho-Cdc2 in DAOY and VC-312 cells at the 15 µM or 25 µM dose of perifosine ($p>0.05$) except 15 µM dose of perifosine decreased phospho-Cdc2 (0.78 SD 0.07) (Figure 3.2).

Figure 3.1 Effect of perifosine on cyclins D in medulloblastoma cells. Cells were treated with 15 µM and 25 µM perifosine for 12 hrs (Figure 3.1A) and 24 hrs (Figure 3.1B). Cells were subsequently harvested and lysed using RIPA buffer. The cell lysates (40 µg) were resolved in NuPage gels and analyzed by Western blotting analysis for Cyclin D1. β-Actin was used as internal control. At both doses of perifosine DAOY cells demonstrated no change in cyclin D1 protein expression on exposure to perifosine whereas VC-312 cells showed a statistically significant increase in Cyclin D1 levels on exposure to perifosine.

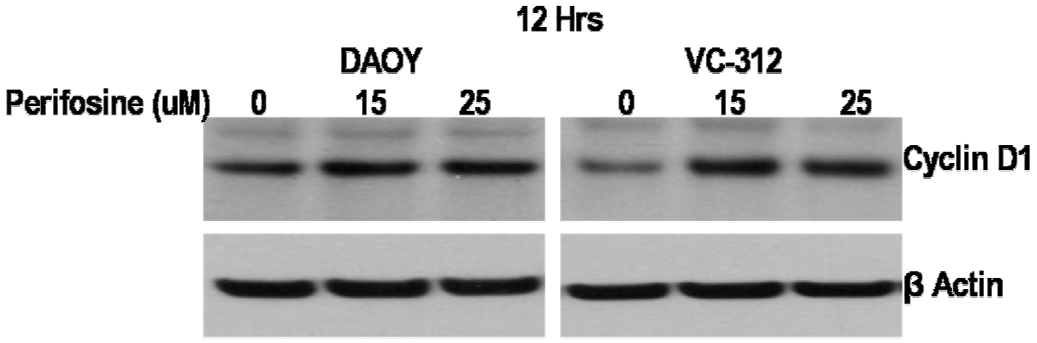

Figure 3.1A

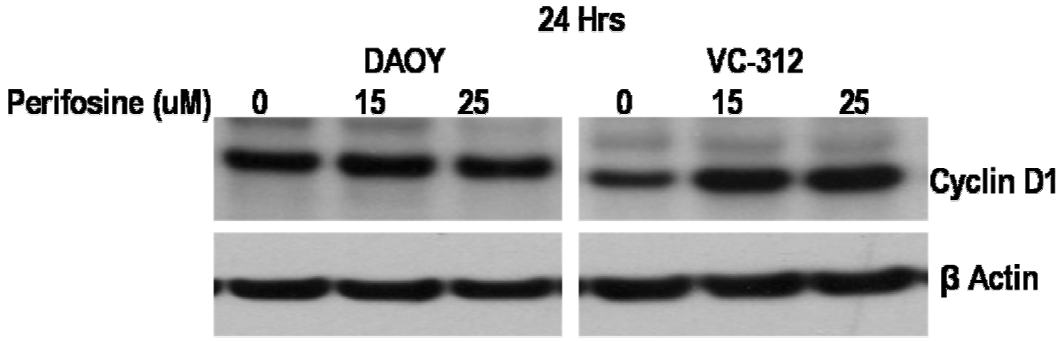

Figure 3.1B

Figure 3.2 Effects of perifosine treatment on cyclin dependent kinase, phospho-Cdc2, in medulloblastoma cells. VC-312 and DAOY cells were treated with 15μM and 25μM dose of perifosine for 24hrs. The cell lysates (40 μg) were resolved in NuPage gels and analyzed by Western blot for phospho-Cdc2. β-Actin was used to ensure equal protein loading. There was minimal change in expression of phospho-Cdc2 in DAOY and VC-312 cells. There was no significant change in the expression of phospho-Cdc2 in DAOY and VC-312 cells in reponse to perifosine ($p>0.05$) except at 15 μM dose of perifosine in DAOY.

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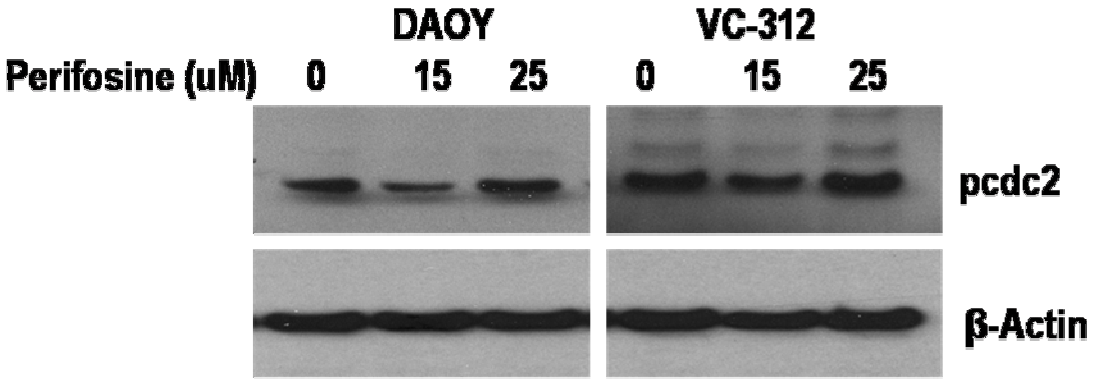

Figure 3.2

Role of Akt mediated cell signaling pathways in perifosine mediated up-regulation of p21$^{waf1/cip1}$

The precise mechanisms by which perifosine treatment increases the expression of proteins such as p21$^{waf1/cip1}$ in medulloblastoma are unclear. Previously we have shown that perifosine decreases the level of phospho-Akt in both medulloblastoma cell lines, DAOY and VC-312. One of the direct substrates for Akt is p21$^{waf1/cip1}$ [146]. p21$^{waf1/cip1}$ is reported to be directly phosphorylated by Akt at two sites (Thr^{145} and Ser^{146}) in the carboxyl terminus. Phosphorylation of Ser^{146} significantly increases p21 protein stability in glioblastoma cell lines [78]. To investigate the association of Akt mediated cell signaling pathway and perifosine-induced p21$^{waf1/cip1}$, we used adenoviral constructs expressing the constitutively active, myristoylated form of Akt, which is not affected by perifosine (Figure 3.3). In a preliminary (one), perifosine (25 μM) treatment led to a 0.15 fold decrease in pAkt and a 0.65 fold decrease in pGSK3 beta expression in untreated control and vector control cells but not in cells expressing constitutively active Akt (Figure 3.3). Perifosine treatment resulted in a 2.5 fold increases in p21$^{waf1/cip1}$ in control cells and a 3.2 fold increase in cells expressing CA-Akt. These results suggest that induced expression of CA-Akt in VC-312 cells overcomes the effect of perifosine on Akt phosphorylation. However, there is still an increase in p21$^{waf1/cip1}$ expression in VC-312 cells expressing CA-Akt. These findings suggest that perifosine-induced up-regulation of p21$^{waf1/cip1}$ may not require a decrease in phosphorylated Akt in medulloblastoma cells. We have not however ruled out the possibility that differences in subcellular localization between the CA-AKT transgene and AKT/ p21$^{waf1/cip1}$ interaction could explain these results. In addition, viral infection may also have an effect on signaling pathways that impact p21$^{waf1/cip1}$.

Figure 3.3 Perifosine induced $p21^{waf1/cip1}$ does not require a decrease in pAkt in medulloblastoma cells. VC-312 cells were infected with adenoviral constructs expressing constitutively active form of Akt (CA-Akt). Cells were subsequently treated with 25μM dose of perifosine for 12 hrs. Cell lysates (40μg) were resolved using NuPage gels and analyzed by western blot for phospho-Serine473-Akt, phospho-Serine9-Gsk3β, $p21^{waf1/cip1}$ and β-Actin using appropriate antibodies. Perifosine treatment led to a decrease in pAkt and pGSK3 beta expression in untreated control and vector control cells but not in cells expressing constitutively active Akt. Perifosine treatment also increased $p21^{waf1/cip1}$ in control cells and cells expressing CA-Akt.

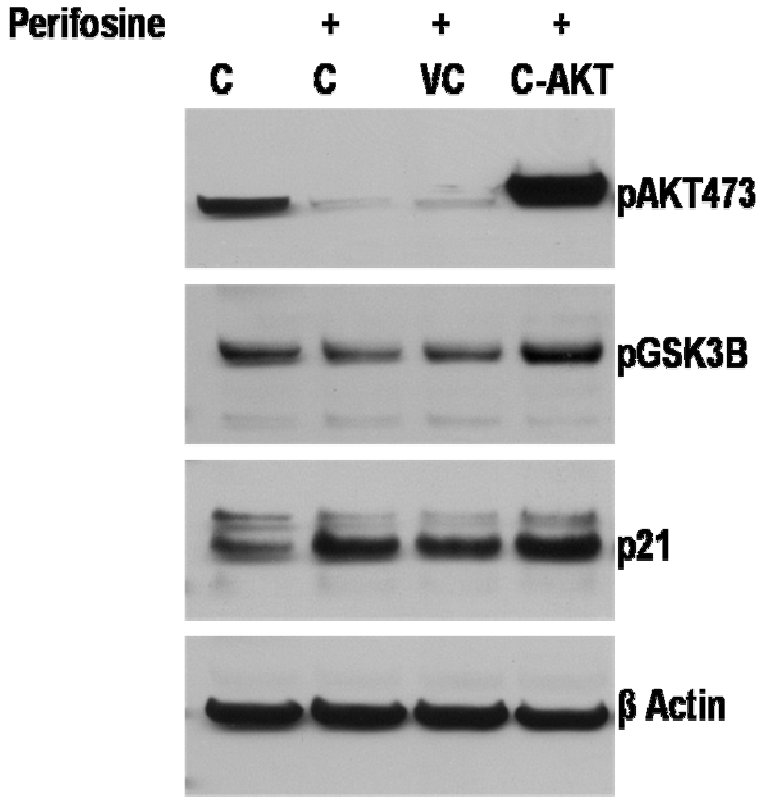

Figure 3.3

Effect of perifosine on MAPK signaling

Cell cycle progression is regulated by distinct cell signaling pathways in cancer cells that include the Akt and MAPK pathways. We have already shown that perifosine decreases phospho-Akt in medulloblastoma cells. Here we determined the effect of perifosine on the MAPK pathway activity by checking the level of phospho-Threonine 204 Erk1/2 (pErk). VC-312 and DAOY cells were treated with 15µM and 25µM dose of perifosine for 12 hours. Perifosine treatment led to a significant increase in pERK in VC-312 cells at 15µM (mean 1.67 fold, SD 0.05) and 25 µM (mean 1.83 fold, SD 0.039) (Figure 3.4). There was a significant decrease at 25µM (mean 0.66, SD 0.063) dose of perifosine in DAOY cells (Figure 3.4). There was no change in total ERK in either cell line with perifosine treatment except in DAOY at 25µM dose (mean 0.87 fold, SD 0.063). Thus, MAPK signaling activation could lead to $p21^{waf1/cip1}$ up-regulation in VC-312 cells. Similar studies done in human keratinocyte cells have shown that activation of ERK by perifosine occurred at concentrations similar to those required for $p21^{waf1/cip1}$ up-regulation, [28].

Figure 3.4 Perifosine induces Erk 1/2 phosphorylation in VC-312 medulloblastoma cells. Cells were cultured for 12 hours in the presence of 15μM and 25μM concentrations of perifosine. Cell lysates were obtained using RIPA buffer. Obtained cell lysates were subjected to Nu-PAGE and electro transferred to nitrocellulose membranes. The membranes were then probed with the anti-pERK, anti-Erk antibodies. β-actin was used as an internal loading control. Perifosine treatment led to an increase in pERK in VC-312 cells at 15 μM and 25 μM perifosine and a decrease at 25μM perifosine in DAOY cells. There was no change in total ERK in VC-312 cells, and no change in DAOY cells at both doses of perifosine.

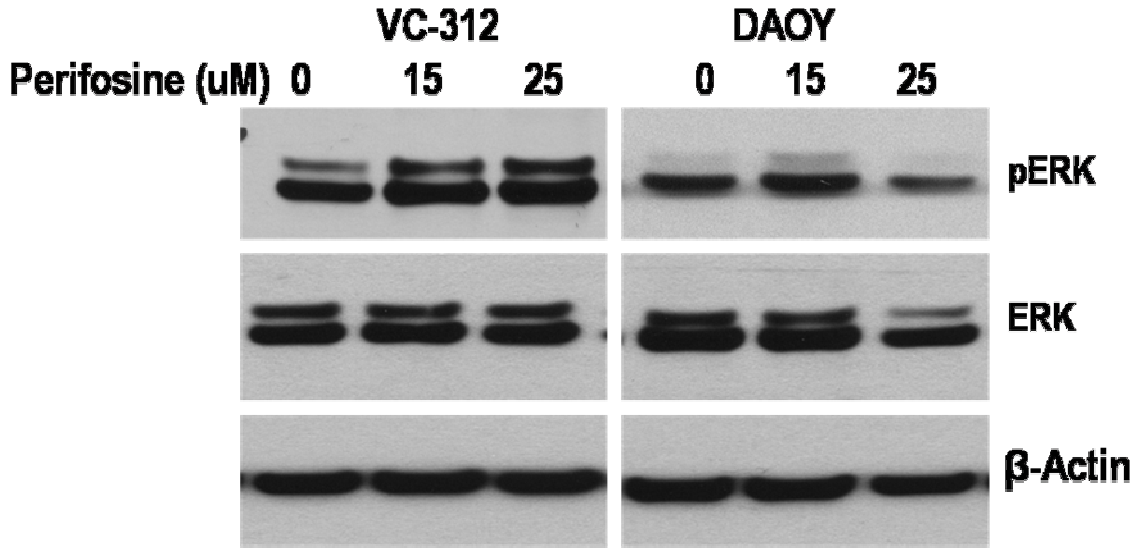

Figure 3.4

Role of MAPK pathway in perifosine-mediated p21$^{waf1/cip1}$ up-regulation

To further investigate the role of MAPK signaling in perifosine mediated up-regulation of p21$^{waf1/cip1}$, we chose VC-312 medulloblastoma cells since perifosine treatment increased the pERK levels in these cells. Cells were first treated with MEK inhibitor (U0126) for one hour, and subsequently with perifosine for 12 hours. MEK inhibitor U0126 decreased the phosphorylation (mean 0.71 fold, SD 0.18) of pERK (Figure 3.5 lane 4) and perifosine alone increased the pERK level at 15μM (mean 1.39 fold, SD 0.19) and 25μM (mean 1.58 fold, SD 0.20) (Figure 3.5 lane 2, 3). Co-incubation of perifosine with MEK inhibitor U0126 (10μM) attenuated perifosine mediated induction of pERK (mean 1.02 fold, SD 0.03) (Figure 3.5 lane 6). MEK inhibitor treatment also increased (mean 5.2 fold, SD 0.36) the expression of p21$^{waf1/cip1}$ at 15 μM dose of perifosine that were effective in inhibiting pERK expression in DAOY cells (Figure 3.5). MEK inhibitor treatment plus perifosine treatment led to a greater increase (mean 18.2 fold, SD 1.79) in the expression of p21$^{waf1/}$, as compared to perifosine alone (mean 11.6 fold, SD 2.81) (Figure 3.5 lane 4). So these findings suggest that inhibition of MAPK signaling through MEK inhibitor alone increases p21$^{waf1/cip1}$ expression in medulloblastoma and treatment with MEK inhibitor plus perifosine has an increased effect on p21$^{waf1/cip1}$ up-regulation versus perifosine alone.

Figure 3.5 Effect of MEK inhibitor U0126 on perifosine induced $p21^{waf1/cip1}$ in VC-312 cells. VC-312 cells were exposed to perifosine in the presence and absence of U0126. Cell lysates were obtained using RIPA buffer. The protein extracts were subjected to Nu-PAGE and transferred to nitrocellulose membranes. The membranes were then probed with antibodies directed at pERK and $p21^{waf1/cip1}$. β -actin was used as an internal control to confirm equal loading in different samples. Inhibition of MAPK signaling using MEK inhibitor increases the expression of $p21^{waf1/cip1}$ in medulloblastoma.

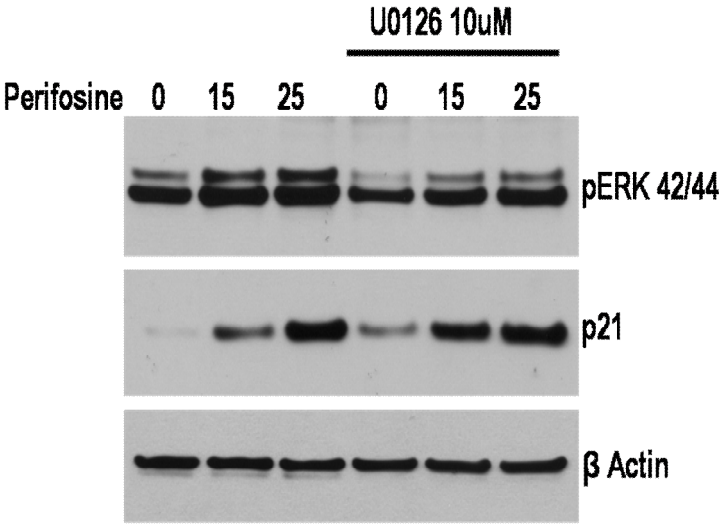

Figure 3.5

Effect of perifosine on $p21^{waf1/cip1}$ and p53 proteins expression

P53 is transcription factor of $p21^{waf1/cip1}$. So, we investigated if perifosine affects p53 protein expression thus explaining the perifosine mediated increase in $p21^{waf1/cip1}$ expression. To determine the effect of perifosine on the cell cycle regulatory proteins p53 and $p21^{waf1/cip1}$ in medulloblastoma cell lines, DAOY and VC-312 cells were treated with perifosine (15, 25 µM) for 24hrs and $p21^{waf1/cip1}$ and p53 levels were determined by western blot. As shown in figure 3.6A, there is a robust increase in $p21^{waf1/cip1}$ protein expression in DAOY at 15 µM (mean 5.28, SD 0.21) and 25 µM (mean 4.82 fold, SD 0.45) of perifosine as shown before in chapter 2. Similarly an increase was found in VC-312 cells at 15 µM (mean 5.08 fold, SD 0.51) and 25µM (mean 10.4, SD 1.42). P53 protein expression appeared to decrease in VC-312 cells at 15 µM (0.80 fold) and 25uM (0.30 fold) and minimally changed in DAOY cells at 15µM (1.02 fold) and 25µM (1.08 fold) with perifosine treatment (data from single experiment, N=1). We also examined the time dependent effect of perifosine (25µM, LD_{50} dose) on $p21^{waf1/cip1}$ and p53 protein expression in VC-312 cells. $P21^{waf1/cip1}$ expression was increased at 3 hours (mean 21.7 fold, SD 2.34) after exposure to perifosine (Figure 3.6B). Based on these preliminary results we determined the effect of perifosine on the p53 level at early time points. In contrast to $P21^{waf1/cip1}$, there was no change in p53 level (mean 0.89 fold, SD 0.36, $p>0.05$) at 3 hours.

Figure 3.6A Effects of perifosine treatment on induction of p53 and p21$^{waf1/cip1}$ in medulloblastoma cells. VC-312 and DAOY cells were treated with perifosine (15µM and 25µM) for 24 hrs. Western blot demonstrated an increase in p21$^{waf1/cip1}$ protein expression at 15µM and 25µM doses of perifosine treatment without any effect on p53 protein level. β-Actin was used to ensure equal protein loading. There was increase in p21$^{waf1/cip1}$ protein expression in DAOY and VC-312 cells at both doses of perifosine. However, perifosine treatment decreases the expression of P53 protein in VC-312 cells and no affect in DAOY cells.

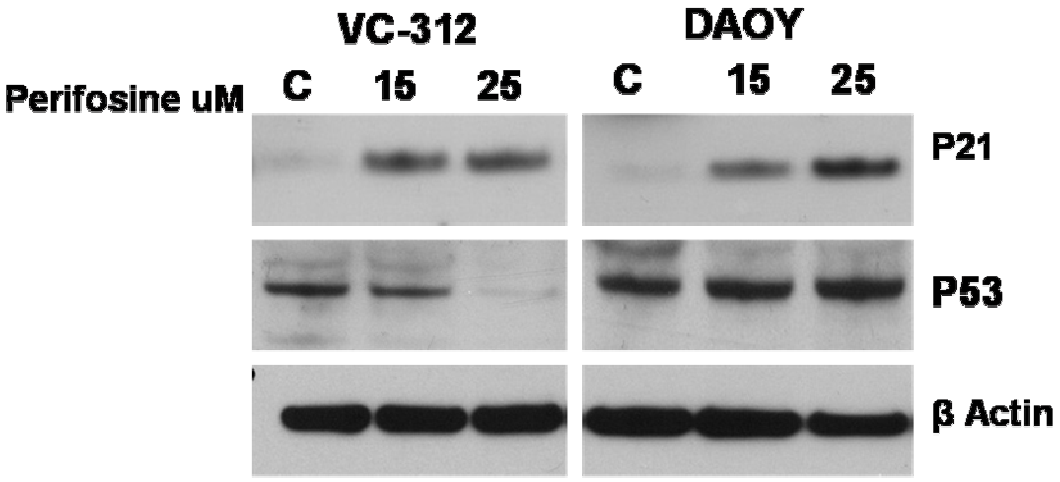

Figure 3.6A

Figure 3.6B Time-dependent effect of perifosine treatment on induction of p53 and p21$^{waf1/cip1}$ in VC-312 cells. VC-312 cells were treated with 25μM perifosine for 24 hrs. P21$^{waf1/cip1}$ protein expression increased at 3hrs after perifosine treatment without any effect on p53 protein level. β-Actin was used as a control for protein loading. P21$^{waf1/cip1}$ expression was increased at 3 hrs after exposure to perifosine. In contrast there was no change in p53 level at 3 hours.

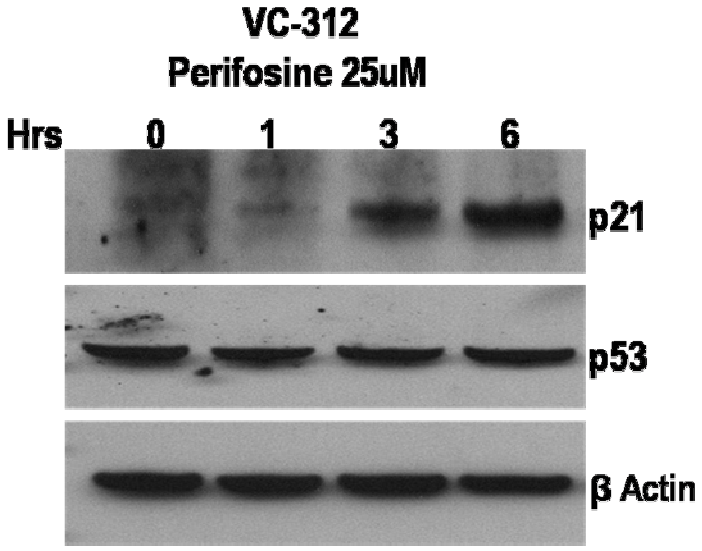

Figure 3.6B

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Discussion

Therapeutic intervention of cancer by molecularly targeted therapies, such as with perifosine, is a new mode of treatment that merits continuing investigation. These therapies can be very effective and less toxic than chemotherapy and radiation therapy. The anticancer potential of perifosine has been studied in various cancer cell models [39, 74, 91]. Findings in the present studies have shown that perifosine has a cytotoxic effect against medulloblastoma cells. These cytotoxic effects could be due to alteration of apoptotic regulatory proteins and induction of cell cycle arrest. In previous studies, we have already shown an effect of perifosine on apoptotic regulatory proteins, however, an effect of perifosine on cell cycle regulatory proteins in medulloblastoma has not previously been established. In this chapter we studied the effect of perifosine on cell cycle regulatory proteins.

The cell cycle is regulated by a family of proteins including cyclin dependent kinases (cdks), cyclins and cdk inhibitors. Cyclin dependent kinases are the main regulators of cell cycle progression, which are positively and negatively regulated by Cyclins (Cyclin D1, D2, D3, E) and cdk inhibitors (p21, p27, p57), respectively. Over-expression of cyclins and loss of cdk inhibitors have been reported in medulloblastoma. Cyclin dependent kinases promote the transcription of genes that are essential for cell cycle by phosphorylating specific target proteins such as Rb. These proteins form complexes with transcription factors and phosphorylation of these proteins releases the transcription factors (E2F), hence increasing transcription of genes [142]. Studies have shown that 22% of medulloblastomas do not express detectable levels of cdk inhibitor p18 [133]. Also knock-out mice that are heterozygous or nullizygous for p27 develop medulloblastoma rapidly if they have another tumor suppressor mutated ("two-hit" tumor suppressor model) [5]. Loss of these cdk inhibitors gives a proliferative advantage to tumor cells

[49]. CDK inhibitors fall into two categories: namely, the INK4 family of proteins (p16/INK4A, p18/INK4C) and the Cip/Kip family (p21$^{waf1/cip1}$, p27/Kip1) [88]. Our results show that perifosine treatment causes an increase in expression of p21$^{waf1/cip1}$, however protein levels of cell cycle regulators p16/INK4A, p18/INK4C and p27/Kip1 were not detected in response to perifosine in medulloblastoma cell lines.

The cell cycle involves sequential phases including G_0, G_1, S, G_2, and M phases. These phases of cell cycle are in turn regulated by two main checkpoints at G_1-S and G2-M. With regard to this aspect, perifosine inhibited cell growth via inducing cell-cycle arrest at G1-S transition in DAOY and at G2-M transition in VC-312. The G1-S and G2-M check points are regulated by Cyclin D, Cyclin B and Cdks. So we studied the effect of perifosine on cyclins and cyclin dependent kinases to determine the possible role of these cell-cycle regulatory proteins in the perifosine mediated effects. Our results demonstrated that perifosine increased Cyclin D1 levels in VC-312 cells without significant effect in DAOY cells. We also determined whether perifosine affected cyclin dependent kinase phospho-Cdc2 (pCdc2) expression. We found no change in pCdc2 in either of the cell lines with perifosine treatment. Additional studies need to be done to further investigate the effect of perifosine on other cyclin dependent kinases.

The studies reported here show that perifosine induces a robust increase in p21$^{waf1/cip1}$ expression in both medulloblastoma cell lines. Increase in p21$^{waf1/cip1}$ has been shown to induce growth cycle arrest and apoptosis in cancer cells [32, 53]. In this report we investigated the mechanisms of perifosine induced p21$^{waf1/cip1}$ up-regulation in medulloblastoma cell lines, which were previously unknown. p21$^{waf1/cip1}$ is a 164 amino-acid protein which belongs to the Cip/Kip family and is encoded on human chromosome 6p21.2 [82]. The N-terminus of p21$^{waf1/cip1}$ contains a domain that binds to cyclin dependent kinases (cdks), some of the main regulatory proteins

involved in the cell cycle. The C-terminal portion of $p21^{waf1/cip1}$ has a nucleus-localizing sequence (NLS), which allows translocation to the nucleus [82]. $P21^{waf1/cip1}$ exists predominantly in a quaternary complex with other regulators of cell cycle (p21/PCNA/cyclin/CDK) [149]. $P21^{waf1/cip1}$ affects the cdks by inhibiting the translocation of cdks into the nucleus and thereby regulating CDK-activating kinase (CAK) activity[149]. The regulation of $p21^{waf1/cip1}$ occurs mainly through transcriptional activation and post-transcriptional modification (mRNA stability). The transcriptional activation of $p21^{waf1/cip1}$ can occur through p53-dependent and p53-independent manners [98]. P53-independent transcription factors include sp1, sp3, smad3/4, p300, Ap2, STAT1/3/5 and P73 [45]. Perifosine has been shown previously to induce $p21^{waf1/cip1}$ by a p53-independent mechanism in other cancer cells [28]. Akt directly phosphorylates $p21^{waf1/cip1}$ at two sites (Thr^{145} and Ser^{146}) in the carboxyl terminus [146]. Phosphorylation of Ser^{146} significantly increases $p21^{waf1/cip1}$ protein stability [78]. Perifosine-mediated accumulation of $p21^{waf1/cip1}$ has been shown to require activation of MAPK signaling, leading to an increase in the affinity and binding of transcription factor Sp1 to the $p21^{waf1/cip1}$ promoter [28].

As perifosine affects cell signaling pathways mediated by Akt and MAPK, we first determined whether these cell signaling pathways are involved in $p21^{waf1/cip1}$ up-regulation. $P21^{waf1/cip1}$ is post-transcriptionally regulated by Akt-mediated cell signaling**.** Perifosine decreased the pAkt in both cell lines. We infected the medulloblastoma cells with an adenoviral construct mediating expression of constitutively active Akt (CA-Akt). CA-Akt contains a myristoylation sequence which has high affinity for the plasma membrane and has been shown to be not affected by perifosine [70]. Similar to earlier studies, perifosine treatment does not decrease pAkt in cells expressing CA-Akt and surprisingly increases the $p21^{waf1/cip1}$. It would be interesting to see the effect of CA-Akt on phospho-$p21^{waf1/cip1}$ in these cells as $p21^{waf1/cip1}$ is a

target of Akt. At the level of transcription, $p21^{waf1/cip1}$ is regulated by MAPK cell signaling and p53 [28, 98]. First we determined the effect of perifosine on p53 protein level. Our preliminary results suggest that perifosine treatment (15μM, 25μM) results in a decrease in p53 in VC-312 at 24 hours and has minimal effect in DAOY (this particular study is currently ongoing and the experiment was done only once). In addition, this study suggests that perifosine treatment does not increase p53 in either cell line. Next we investigated the role of the MAPK signaling pathway in perifosine induced $p21^{waf1/cip1}$ up-regulation. Perifosine treatment has been shown to increase expression of proteins in the MAPK pathway [28, 64, 97]. Consistent with previous observations, there was a marked increase in pERK with perifosine treatment in VC-312 medulloblastoma cells. We sought to determine whether the induction of pErk in VC-312 cells was associated with perifosine $p21^{waf1/cip1}$. Perifosine mediated up-regulation of pErk was blunted with MEK inhibitor U0126. Surprisingly, the expression of $p21^{waf1/cip1}$ was increased in VC-312 cells with the combined treatment of perifosine and U0126 as compared to treatment with perifosine alone. These findings suggest that perifosine mediated up-regulation of $p21^{waf1/cip1}$ involves components other than pAkt and the MAPK pathway. Further studies need to be done to investigate the role of other proteins.

CHAPTER 4

GENERAL DISCUSSION

Medulloblastoma is one of the most common embryonic tumors in children. Surgery, radiotherapy and chemotherapy are standard treatment modalities for medulloblastoma tumors. There had been recent advancements in chemotherapy but these chemotherapeutic drugs still have suboptimal effectiveness due to chemo-resistance. A more complete understanding of the molecular mechanisms that underlie drug resistance in medulloblastoma will help in the development of better therapeutic strategies for these tumors. These tumors are formed during brain development. Normal development of brain involves proliferation, differentiation and migration of neurons and their supporting structures during prenatal and postnatal life [47]. These cellular processes are maintained by cell signaling pathways via interaction of cells with each other. Disruption of normal signal transduction impairs normal development of the brain and can lead to hyper-proliferation and eventually tumor formation [114]. Mechanisms of disruption of these cell signaling pathways involve chromosomal loss and gene amplifications [22, 23, 100]. Akt kinases represent one of the main regulatory elements involved in cell signaling pathways and aberrant activation of this pathway is thought to be a common underlying factor in the pathogenesis of human malignancies including medulloblastoma [60]. Targeting Akt pathway with molecular targeted therapy has been shown to markedly reduce tumorigenicity in some cancer cells [19]. In this study, we determined the effect of inhibiting Akt mediated cell signaling on medulloblastoma cell survival and chemo-resistance.

Akt is a 57 KD protein, present in three structurally homologous isotypes, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). The N-terminus of all Akt isoforms contains the PH domain which has similar specificities for the D3-phosphorylated phosphoinositide on the cell membrane which is required for their activation [40]. The expression of these isoforms varies in different tissues. Akt 1 and Akt 2 are universally expressed in all cell types whereas Akt3 is restricted to

the brain and testis [40]. Akt kinases are part of a survival pathway that has been found to be involved in chemo-resistance. We first characterized the isoform expression of Akt proteins in medulloblastoma cell lines DAOY and VC-312. All Akt isoforms (Akt1, Akt2, and Akt3) were detected in both cell lines (Figure 2.1A). We also compared the mRNA levels of Akt isoforms in medulloblastoma tumor samples to those in normal brain. The mRNA of Akt2 was significantly higher in medulloblastoma tumor samples as compared to normal cerebellum (Figure 2.1B). The Akt2 gene has been frequently observed to be over-expressed and/or amplified in other tumors, including human pancreatic and ovarian carcinomas [17, 110]. The mechanisms underlying Akt isoform functional specificity derive partly from their sub-cellular distribution [50]. Despite differences in structure, the fundamental mechanisms of activation of all isotypes are the same. Each isotype has different specificity for different sub-cellular compartments which contributes to the specificity of Akt isotype functions. Akt isotypes function by phosphorylation of its downstream substrates which is consistently associated with tumor formation, proliferation and chemo-resistance [18, 25]. In this study also, we also found increased expression of phosphorylated Akt in medulloblastoma clinical samples as compared to the normal brain.

Factors that drive the activation of these Akt isotypes in medulloblastoma still remain to be elucidated; however there is evidence that supports decreased expression of PTEN, a negative regulator of Akt signaling in medulloblastoma. Currently, one proposed mechanism for decreased PTEN expression is hypermethylation of its promoter [60]. We hypothesize that the Akt pathway would also be involved in medulloblastoma cell survival, proliferation and chemo-resistance. The Akt N-terminal PH domain is 60%-80% homologous among all Akt isotypes and explains the similarity in how they are activated. The activated Akt then phosphorylates target proteins involved in tumor cell survival and proliferation [18]. Inhibition of this pathway has been

found to induce cell death in a variety of tumor cells [16, 110]. To determine the role of Akt kinase in medulloblastoma, pAkt (active Akt) levels were first determined using western blot analysis of medulloblastoma cell lines derived from primary tumors. These cell lines demonstrate expression of both pAkt and the inactive Akt (Figure 2.1A). We then determined the effect of inhibition of Akt mediated cell signaling in medulloblastoma cells, downregulating Akt by pharmacological and genetic methods. We chose the pharmacological route using a novel phospholipid analogue, perifosine that has been shown to inhibit Akt by inserting itself into the cell membrane and inhibiting PH domain-mediated activation. This drug is currently undergoing phase I and phase II clinical evaluation in advanced solid tumors including colon and ovarian carcinomas [24, 74]. The effect of perifosine was determined on DAOY and VC-312 medulloblastoma cells by exposing them to a single treatment of the drug for 24 hrs. Perifosine treatment was found to decrease the cell viability in both medulloblastoma cell lines in a dose-dependent manner (Figure 2.3). A steep decline in cell viability was noted at doses ranging from 15uM to 35uM in both cell lines, and the LD_{50} was established at approximately 25uM (Figure 2.3). This LD_{50} dose of perifosine at 24 hrs is higher than the maximum achievable plasma concentration of perifosine in tumor patients (19uM) [24]. It is important to note that while in published *in vivo* studies 19uM doses of perifosine was maintained for extended durations (weeks) whereas our LD_{50} dose was calculated by exposing these cells for 24 hrs. In subsequent experiments, we calculated the LD_{50} dose of perifosine at extended time points- 72hrs and 120hrs. The LD_{50} of perifosine calculated at 72 hrs and 120 hrs and was found to be approximately 15uM and 10uM respectively (Appendix A), which is less than the maximum achievable plasma concentration in patients. These findings suggest that either perifosine has a long half life in medulloblastoma cells and may act for an extended period or that it initiates a

fatal, self-renewing cascade that eventually leads to a decrease in cell viability. Perifosine treatment resulted in a significant decrease in pAkt along with a decrease in cell viability (Figure 2.2, 2.3) and had no effect on total Akt. In subsequent experiments we also determined the time-dependent effects of perifosine on medulloblastoma cells and noted a complete loss of detectable phospho-AKT in both cell lines at this concentration by 6 hours following treatment (Figure 2.4A).

The decrease in pAkt was found to precede the decrease in cell viability, indicating a temporal relationship between the events. These experiments suggest that Akt signaling could be involved in medulloblastoma cell survival. Studies have been conducted in different cancer types including prostate cancer cells to assess if perifosine-induced cytotoxicity involved Akt mediated cell signaling. Constitutive activation of Akt (CA-Akt) protects prostate cancer cells from perifosine-induced cell death [69]. CA-Akt possesses a myristoylated sequence at the N-terminus that has a higher affinity for the cell membrane and as a result of this the translocation of CA-Akt from cytosol to cell membrane is not inhibited by perifosine. Preliminary experiments were conducted in which VC-312 cells were transfected with CA-Akt and it was found that this can override perifosine-mediated decrease in pAkt in medulloblastoma cells (Figure 3.3).

A caveat of targeting Akt using perifosine include potential off target effects on other cell signaling pathways, such as the MAPK pathway, which may also be initiated by components of the cell membrane. The MAPK pathway is also known to regulate cell proliferation and survival and, is found to be active in medulloblastoma [144]. We sought to determine if perifosine also had an effect on these pathways. Interestingly, we found that the impact of perifosine on MAPK signaling differs in the cell lines- DAOY and VC-312. One of the main proteins involved in

MAPK signaling is Erk and activation of MAPK signaling was confirmed by detecting pErk with immunoblot. Perifosine treatment increased pErk in VC-312 cells at doses of 15uM and 25uM, while it had no effect on the DAOY cells (Figure 3.4). These results indicate that MAPK signaling is induced in VC-312 medulloblastoma cells in response to perifosine and could be explained by a decrease in pAkt due to perifosine since the two pathways negatively modulate each other [61, 109]. Despite differential effects on MAPK signaling in DAOY and VC-312 cell lines, the LD_{50} dose of perifosine for cell viability in both cell lines is the same. This suggests that MAPK signaling is unlikely to be a major component in mediating the effect of perifosine on cell viability in medulloblastoma cells.

After establishing that perifosine decreases cell viability in medulloblastoma cells, we explored the mechanism of cell death in response to perifosine. In general, tumor survival and proliferation depends upon deregulation of cellular events such as cell cycle progression and apoptosis [62, 83]. Akt pathway directly impinges upon these cellular events, and we sought to determine the effects of perifosine on apoptosis and the cell cycle [33, 79]. Apoptosis is best described as programmed cell death. It involves a complex set of events within a cell leading to activation of proteolytic enzymes known as caspases which then act in concert to cause morphological changes in the cell, ultimately leading to cell death [33]. We examined the effect of perifosine on different caspases in medulloblastoma cells. Treatment with perifosine at the LD_{50} dose (25uM) was found to trigger cleavage of caspases resulting in induction of apoptosis. In both cell lines, caspase-3 and caspase-9 cleavage was detected after 6 hrs (Figure 2.4A, 2.4C). Notably, these were also the time points associated with a decrease in cell viability. The activation of these caspases either requires loss of mitochondrial integrity leading to release of cytochrome C (intrinsic apoptotic pathway) or activation of Fas/CD95 death receptor on the cell

membrane (extrinsic apoptotic pathway). Perifosine induces release of cytochrome C from mitochondria and also recruits Fas/CD95 death receptor to the cell membrane in multiple myeloma cancer cells [44]. These active caspases directly cleave a target protein, PARP. In this work PARP cleavage was detected upon exposure to perifosine in both medulloblastoma cell lines examined (Figure 2.4B). Results from these studies indicate that the response to perifosine includes a dramatic increase in caspase activity in medulloblastoma cells.

Having establishing the role of perifosine in the intrinsic pathway, we set out to determine if it would affect the extrinsic pathway as well. In acute myelogenous leukemia cells, perifosine activates the extrinsic pathway of apoptosis by increasing the expression of death receptors DR4/DR5 on the cell surface [130], which then activates caspase 8, ultimately leading to apoptosis [20]. We did not observe any caspase-8 cleavage in medulloblastoma cells following treatment with perifosine. Interestingly, 62% of medulloblastoma tumors have inactivation of the caspase-8 gene by aberrant methylation of its promoter [51]. This might account for our finding of no increase in caspase-8 following exposure to perifosine as these cells may be inherently incapable of synthesizing this protein.

The aforementioned results suggest that perifosine mediates cell death in medulloblastoma by activating the intrinsic pathway of apoptosis. However, perifosine might cause cell death independent of caspase-mediated apoptosis. Perifosine has been shown to induce caspases independent of cell death by increasing JNK and c-jun phosphorylation in acute lymphoblastic cells [20]. Thus, it will be important in future studies to explore other mechanisms of cell death in response to perifosine treated medulloblastoma cells.

Tumor cell growth can be affected not only by cytotoxic mechanisms but also by cytostatic mechanisms. With this in mind, we investigated if perifosine would have a cytostatic

effect on medulloblastoma cells. Cells were stained with propidium iodide and examined using flow cytometry for cell cycle changes. Quantitative analysis of flow cytometric results showed that treatment with perifosine led to cell cycle arrest in both cell lines. Interestingly, perifosine induced cell cycle arrest at the G1/S phase in DAOY and at the G2/M phase in VC-312 cells (Figure 2.6). Thus, perifosine acts to halt cells in different phases of the cell cycle in the two medulloblastoma cell lines studied. We therefore explored the mechanism of cell cycle arrest in these cells in order to better understand these differences.

In normal cells, cell cycle is tightly regulated by cyclin dependent kinases (cdks). Activity of these cdks is controlled by cyclins (positive regulators) and cdk inhibitors (negative regulators) [132]. While activation of the cdks helps the cell to move from one phase of the cycle to another, over-activation leads to an uncontrolled cellular proliferation which might be a key factor in tumorigenesis[49]. Furthermore, loss of cdk inhibitors has been found in various malignancies, and this also can lead to uncontrolled cell cycle progression and cancer growth [120]. We determined the effect of perifosine on cyclins and cdk inhibitors in medulloblastoma cells. Perifosine has been shown to induce $p21^{waf1/cip1}$ (one of the cdk inhibitors) in squamous carcinoma cells [104]. Similarly, perifosine induced $p21^{waf1/cip1}$ (at the doses of 15uM and 25uM) in medulloblastoma cell lines (Figure 2.7). Importantly, silencing of $p21^{waf1/cip1}$ with siRNA in VC-312 cells overcame the cell cycle arrest induced by perifosine (Figure 2.8B). This indicates a potential role for $p21^{waf1/cip1}$ in perifosine induced cell cycle arrest in medulloblastoma cells. Up-regulation of $p21^{waf1/cip1}$ can also influence the cell's abilities to undergo differentiation and apoptosis, in addition to promoting cell cycle arrest [32, 53]. Thus $p21^{waf1/cip1}$ up-regulation in response to perifosine might be responsible for cytotoxic and cytostatic effects in medulloblastoma. The endogenous expression of $p21^{waf1/cip1}$ in medulloblastomas is low, as its

negative regulator, FOXG1 is amplified in a majority of these tumors [2]. After determining the effect of perifosine on cdk inhibitors, we attempted to examine the effect of perifosine on cyclins, another group of cell cycle regulators. We had already established that perifosine treatment induces cell cycle arrest at the G1/S phase of the cell cycle in DAOY and the G2/M phase of cell cycle in VC-312. Consequently, we first turned our attention to the effect of perifosine on the cyclins involved in these two phases of the cell cycle. Cyclins interact with cdk and drive the progression of a cell through different phases of the cell cycle. The main cyclins involved in the above-mentioned cell-cycle phases are cyclin D (G1/S) and cyclin B (G2/M) [132]. The expression of these cyclin proteins increases in response to mitogenic stimuli [71], and their binding to Cdks leads to their activation. Activated cdks then phosphorylate target proteins and increase the expression of genes required for cell cycle progression [83]. We could not detect cyclin B with western blot analysis in either cell line. Perifosine treatment induced cyclin D1 in VC-312 without any effect in DAOY cells (Figure 3.1). As previously mentioned, perifosine treatment also led to an increase in the expression of pERK in VC-312 cells without any effect in DAOY cells. It would be interesting to see if there is any association between perifosine induced pErk and cyclinD1 in VC-312 cells. CyclinD1 expression has been found to be induced in response to activation of MAPK signaling (increase in pERK) [68]. Another notable finding was that there was increase in expression of $p21^{waf1/cip1}$ (negative regulator of cell cycle) and cyclinD1 (positive regulator of cell cycle) with perifosine treatment in VC-312 cells. These two proteins compete with each other to influence the levels of cdks. $p21^{waf1/cip1}$ is a universal cdk inhibitor and can affect any cdk whereas cyclin D1 selectively affects cdks involved in the G1/S phase of cell cycle. Thus, induction of cyclin D1 protein in response to perifosine in VC-312 might increase the cdk activity involved in G1/S phase of cell cycle, which might rescue VC-312

cells from arresting in G1/S phase of cell cycle as compared to DAOY medulloblastoma cells. Perifosine induced $p21^{waf1/cip1}$ would then inhibit the cdks involved in the next phase of the cell cycle (G2/M), leading to cell cycle arrest in G2/M in VC-312 cells.

Next we investigated the mechanism of perifosine-mediated $p21^{waf1/cip1}$ up-regulation in these medulloblastoma cells. In prostate cancer cells, perifosine is known to induce $p21^{waf1/cip1}$ by down-regulating p-Akt, while expression of CA-Akt abrogates perifosine-mediated decrease in p-Akt and up-regulation of $p21^{waf1/cip1}$ [69]. In contrast to these findings, our studies showed that CA-Akt expression did not have any effect on perifosine induced $p21^{waf1/cip1}$ expression (Figure 3.3). $p21^{waf1/cip1}$ is transcriptionally regulated by p53 and MAPK signaling pathway components. We investigated the role of the MAPK signaling pathway in perifosine induced $p21^{waf1/cip1}$ up-regulation. Induction of MAPK signaling increases the activity of transcription factors like Sp1, leading to increased expression of $p21^{waf1/cip1}$ [64, 97]. Perifosine treatment led to an increase in pErk levels (MAPK) in VC-312 cells (Figure 3.4). To determine the role of MAPK signaling in perifosine induced $p21^{waf1/cip1}$, we blunted the up-regulation of pErk using the MEK inhibitor U0126 and then assessed the effect of perifosine on $p21^{waf1/cip1}$ protein level. Blunting of pErk did not attenuate perifosine mediated $p21^{waf1/cip1}$ expression (Figure 3.5). Surprisingly, the expression of $p21^{waf1/cip1}$ was increased in VC-312 medulloblastoma cells treated with perifosine and MEK inhibitor as compared to treatment with perifosine alone. This paradoxical finding suggests that perifosine mediated up-regulation of $p21^{waf1/cip1}$ does not appear to involve up-regulation of pErk in VC-312 medulloblastoma cells. Further studies are warranted to definitively exclude the role of MAPK pathway in perifosine induced $p21^{waf1/cip1}$ by transfecting these cells with dominant negative MEK (DN-MEK) or siRNA directed against MEK.

P53 is transcription factor for $p21^{waf1/cip1}$. To investigate whether $p21^{waf1/cip1}$ up-regulation is related to p53, we determined the effect of perifosine on p53 protein expression and found no increase in p53 protein expression with exposure to perifosine (15uM, 25uM) in either cell line (Figure 3.6A, 3.6B). In this study we only determined the effect of perifosine on p53 protein level and did not examine the effect of perifosine on p53 transcriptional activity. The effect of perifosine on p53 functional activity would nevertheless be an interesting future study.

As mentioned above, perifosine acts on medulloblastoma cells by inducing apoptosis and cell cycle arrest. Similar effects have been reported in human gastric cancer cells in response to chemotherapy [80]. The signaling mechanisms associated with cell cycle arrest and apoptosis are interconnected and thus influence each other. Cell cycle arrest can induce apoptosis by decreasing the level of anti-apoptotic proteins as during cell cycle arrest, there is dissociation of transcription from translation because of loss of the nuclear membrane [11]. This would result in a decrease of proteins and mRNAs with short half-lives such as Mdm-2, XIAP, cIAP-2 and FLIP all of which have anti-apoptotic functions. The relative deficiency of these anti-apoptotic proteins triggers apoptosis. It would be interesting to determine the effect of perifosine on these proteins in DAOY and VC-312 medulloblastoma cell lines.

In this study, we only report the effect of perifosine on apoptosis and cell cycle. Another mechanism which might contribute to perifosine-mediated cell death in these cells is autophagy. In this process the cell degrades its own components through the lysosomal machinery. This usually occurs in terminally differentiated cells such as cardiomyocytes and neurons [137]. Regulation of autophagy involves autophagosome formation and is negatively regulated by PI3K/Akt signaling through the mammalian target of rapamycin (mTOR). Perifosine-mediated inhibition of Akt signaling might increase autophagosome formation leading to autophagy in

these cells. It would be interesting to determine if perifosine-induced cell death involves autophagy in these cells.

Perifosine targets cell membranes and interferes with signal transduction pathways. Due to this distinct mode of action, this drug is an attractive candidate to combine with chemotherapy and radiotherapy to overcome therapeutic resistance [139]. We tested the effect of perifosine on etoposide- and radiation-induced cell death in these cell lines. Perifosine enhanced etoposide and radiation-induced cell death. This effect was additive for VC-312 and synergistic for DAOY, and resulted in a marked increase in cell death (Figure 2.5A, 2.5B). The apparent synergy seen in the current study was observed at clinically achievable concentrations of perifosine (10uM).

Our studies have thus far mainly focused on *in vitro* techniques to elucidate the effects of perifosine on medulloblastoma cell lines, its effects in the *in vivo* setting remains to be determined. Perifosine is a highly lipophilic agent and has been demonstrated to cross the normal blood-brain barrier (BBB) in rodents [24, 138]. Also, brain tumors have an imperfectly formed or "leaky" BBB, which would allow greater access to the tumor bed. Hence, use of perifosine for *in vivo* tumor models might very well be feasible and forms an important aspect of our future studies. In summary, perifosine appears to be a promising adjuvant treatment, deserving of further evaluation in pediatric brain tumors such as medulloblastoma.

Conclusion:

In summary, medulloblastoma (MB) is the most common malignant cancer of the central nervous system in children. Akt kinases are critical for tumor cell survival and proliferation and have been found to be significantly elevated in medulloblastoma cells. Perifosine, an inhibitor of Akt kinase, induces a rapid decrease in cell viability with pronounced suppression of

phosphorylated Akt. Perifosine acts on medulloblastoma cells by inducing apoptosis and cell cycle arrest, similar to other chemotherapeutic drugs in cancer cells [80]. Apoptosis and cell cycle arrest are mediated by activation of caspases and $p21^{cip1/waf1}$ proteins respectively. Specifically, apoptosis was affected by the intrinsic pathway, while cell cycle arrest was found to be mediated by up-regulation of the cdk inhibitor- $p21^{waf1/cip1}$. Up-regulation of $p21^{waf1/cip1}$ has been shown to increase cancer cell's ability to undergo differentiation, apoptosis and cell cycle arrest. $p21^{waf1/cip1}$ expression can be regulated by p53, Akt and MAPK pathways. Perifosine has been shown to up-regulate $p21^{waf1/cip1}$ by a p53 independent mechanism, via up-regulation of the MAPK pathway in other cancer cells. In the present study, we did not find any increase in p53 protein levels in response to perifosine. In contrast to earlier studies, MAPK inhibition resulted in a paradoxical increase in $p21^{waf1/cip1}$ expression. Thus, perifosine induced increase in $p21^{waf1/cip1}$ does not appear to be mediated by p53 or MAPK signaling in the medulloblastoma cell lines examined herein, suggesting the involvement of an alternative pathway or pathways.

These studies confirm that perifosine inhibits Akt pathway in medulloblastoma cells and targets key regulatory proteins involved in apoptosis and the cell cycle. In other studies, activation Akt pathway has shown to bestow therapeutic resistance in cancer cells [86], as a result perifosine can have significant potential as a new effective therapy in medulloblastoma. Perifosine, when used in combination with existing modalities of treatment for medulloblastoma (i.e. sub-lethal doses of etoposide or irradiation), significantly enhances the efficacy of the latter. These findings indicate that perifosine, either alone or in combination with other treatment methods, might be an effective therapeutic agent for the treatment of medulloblastoma.

We determined the effect of another Akt inhibitor on medulloblastoma cells. Akti IV is another potent Akt inhibitor, which decreases cell viability. Perifosine and Akti IV have different mechanisms of action to suppress Akt. The combined effect of these two Akt inhibitors was more than additive on reducing cell viability in medulloblastoma cells at lower doses in preliminary studies (Appendix D). The combination of these two possibly enhances the inactivation of Akt (although not examined in study) leading to decrease in cell viability. We also determined the effect of perifosine on another pediatric brain tumor, ependymoma. Surprisingly, in this study, ependymoma cells were not as sensitive as medulloblastoma cells (Appendix C). In addition, perifosine treatment did not decrease pAkt expression in ependymoma cells at concentrations up to 100 μM. These results suggest that the perifosine effect on pediatric brain tumors may be tumor cell type specific.

Future Directions

Akt has been recognized as a key component in tumor cell proliferation. Akt has three isoforms and each isoform has specific functions: Akt1 is involved in cell growth, Akt2 in maintaining glucose homeostasis and Akt 3 in cell growth mainly restricted to the brain [16, 59]. Targeting Akt using perifosine would nonspecifically inhibit all isoforms as they all have the same mechanism of activation. Future studies will determine the effect of knock down of each of these isoforms using specific synthetic small-interfering RNAs or monoclonal antibody in different tumors. Preliminary studies have shown that knocking down each isoform of Akt affects the growth of cancer cells [59], indicating the potential role of specific Akt isoforms in cancer cell survival. Perifosine has been used in vivo in animal models of some other cancer cells[15]. Future studies are needed to see if perifosine's *in vitro* effects in medulloblastoma cells can be reproduced in *in*

vivo tumor models. Our lab has recently generated tumors in nude mouse brains using VC-312 cells. Perifosine can be used in these nude mice with a loading dose of 300 mg/kg and maintenance dose of 35 mg/kg by oral route, similar to a study done in glioma cells[27]. This study will potentially argue for the use of perifosine in medulloblastoma patients.

Mechanism of perifosine induced increase in $p21^{waf1/cip1}$ is still unknown in medulloblastoma cells. FOXG1 protein is a negative regulator of $p21^{waf1/cip1}$ which maps to chromosome 14q. Sixty seven percent of medulloblastoma tumors are found to have gain of chromosome 14q [2]. Quantitative real time PCR showed a 2-7-fold copy gain for FOXG1 in medulloblastoma tumor cells. Decrease in expression of FOXG1 in DAOY cell line using siRNA up-regulates $p21^{waf1/cip1}$ [2]. It would be interesting to see the effect of perifosine on FOXG1 protein in medulloblastoma cells and if FOXG1 mediates perifosine induced $p21^{waf1/cip1}$ upregulation.

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APPENDIX A

Effect of perifosine on medulloblastoma cells at extended time points

Rationale

Perifosine is very effective in reducing the cell viability in medulloblastoma cells. The LD_{50} dose of perifosine in medulloblastoma cell lines was determined to be 25μM at 24 hrs. This LD_{50} dose of perifosine is higher than the maximum achievable plasma concentration of perifosine in tumor patients (19uM) [24]. However, it is important to note that the maximum achievable plasma concentration of perifosine can be maintained clinically for weeks, while our LD_{50} was calculated by exposing these cells for only 24 hrs. We therefore set out to determine the LD_{50} of perifosine at more extended time points- 72hrs and 120hrs.

Methods

The medulloblastoma cell line VC-312 were plated in white, opaque-walled, sterile, 96-well plates at the density of 1000 cells per 100 μL of growth medium per well. Cells were treated with perifosine (10μM and 20μM) versus vehicle control (PBS) for 24, 72, 120 hours. Cell viability was determined using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI). The cell viability was calculated with the following equation: % Survival = (x/y)*100 where 'x' is the number of viable cells in conditions exposed to perifosine, 'y' is number of cells treated with vehicle control.

Results and discussion

We found a significant decrease in cell viability of VC-312 cells treated with 10μM and 20μM perifosine at 72hrs and 120 hrs compared to 24 hrs ($P< 0.05$) (Figure A). The LD_{50} of

perifosine calculated at 72 hrs and 120hrs was found to be approximately 15uM and 10uM, which is less than the maximum achievable plasma concentration in patients. These findings suggest that perifosine can have cumulative effects on growing cells over extended time periods.

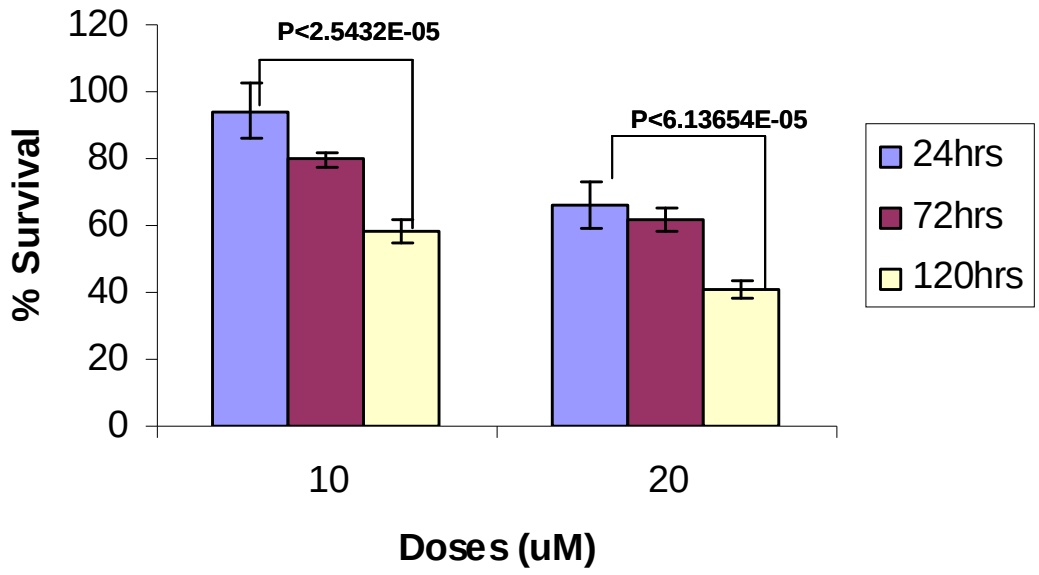


Figure A

APPENDIX B

The effect of caspase inhibitors on perifosine-induced decrease in cell viability

Rationale

Perifosine decreases medulloblastoma cell viability by inducing caspase cleavage in these cells. To further determine the potential role of caspase activation in perifosine mediated cell death, this study was performed using a selective small molecule inhibitor of caspase 3 (obtained from EMD Biosciences).

Methods

Cell viability was determined using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI). VC-312 cells were plated in white, opaque-walled, sterile, 96-well plates at the density of 1000 cells per 100 µL of growth medium containing 10% FBS at 37 °C overnight. After 24 hours, cells were first treated with caspase3 inhibitor (from Calbiochem/EMD bioscience) (10, 25, 50 µM) for one hour and then with increasing concentrations of perifosine (10µM to 50µM) for 24 hours. The cell viability was calculated with the following equation: % Survival = (x/y)*100 where 'x' is the number of viable cells in conditions exposed to perifosine, caspase 3 inhibitor or both, 'y' is number of cells treated with vehicle control

Results and discussion

We observed that the caspase3 inhibitor did not affect the cell viability at lower doses of perifosine (10, 20 µm). Higher doses of caspase3 inhibitor partially attenuated the effect of higher dose of perifosine. Maximum attenuation was seen with 50 µM caspase inhibitor and 62 µM perifosine (Figure E) ($P < 0.05$). On x axis labeled P is perifosine and C is caspase 3 inhibitor.

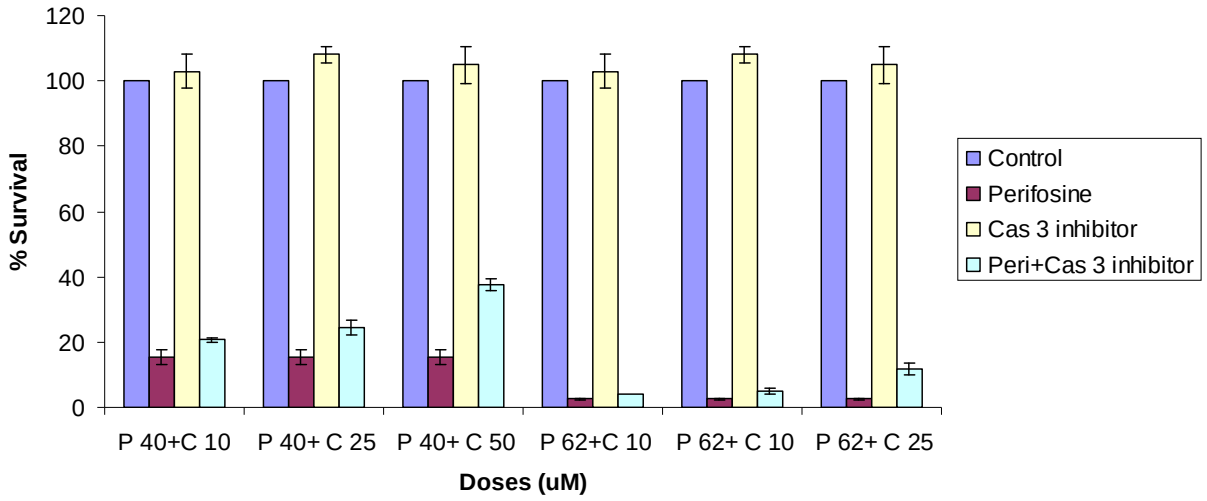


Figure B.

APPENDIX C

Differential response of pediatric brain tumor cells to perifosine

Rationale

Perifosine is very effective in reducing the cell viability of medulloblastoma cells. We examined the effect of perifosine on another common pediatric brain tumor, ependymoma. This study compared the effects of perifosine on cell viability and cell signaling pathways in medulloblastoma cell lines and ependymoma cell line.

Methods

The human ependymoma-derived cell line R196 was obtained from American Type Culture Collection (ATCC). These cells were plated in white, opaque-walled, sterile, 96-well plates at the density of 1000 cells per 100 µL of growth medium per well. Following 24 hour incubation, cells were treated with perifosine (1µM to 100µM) versus vehicle control (PBS) for 24 hours. Cell viability was determined using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI).

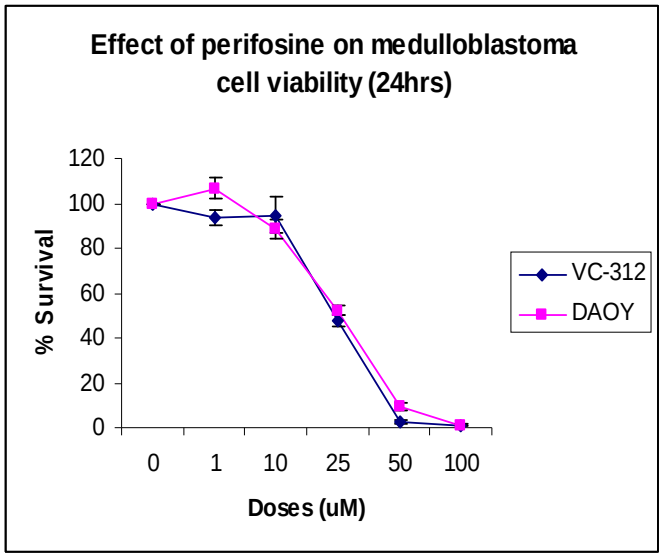
Western Blot

Cell lysates were prepared from cells treated with perifosine (1um to 100uM). The proteins were resolved on Novex NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes at 35 V for 2 hours (Invitrogen). The membranes were incubated with pErk and pAkt antibodies. Western Blots were developed using the ECL Detection System (GE

Healthcare-Amersham Biosciences, Piscataway, NJ, USA). β-Actin antibody (1:5,000, Sigma Biotechnology) was used as a control for protein loading.

Results and discussion

We examined the effect of perifosine treatment on the viability of medulloblastoma (VC-312, DAOY) and ependymoma (R196) cells. Perifosine induced a dose dependent decrease in cell viability in medulloblastoma cell lines, shown in figure C1. The LD_{50} (lethal dose to 50%) for DAOY and VC-312, determined using 3 replicate viability assays, was 25 μM (Figure C1). Surprisingly, ependymoma cell viability did not decline in response to perifosine at lower doses. There is a steep decline in cell viability at doses of perifosine higher then 50 μM in ependymoma cells. We also compared the effect of perifosine on pAkt and pErk in medulloblastoma (VC-312) cells and ependymoma (R196) cells. Western blot analysis showed a decrease in pAkt (mean 0.12 fold) and an increase in pErk (mean 4.2 Fold) with perifosine treatment (100uM) in VC-312 cells (figure C2). In ependymoma cells (R196) perifosine treatment led to a decrease in pErk (mean 0.22 fold) and had minimal effect on pAkt levels (figure C2).



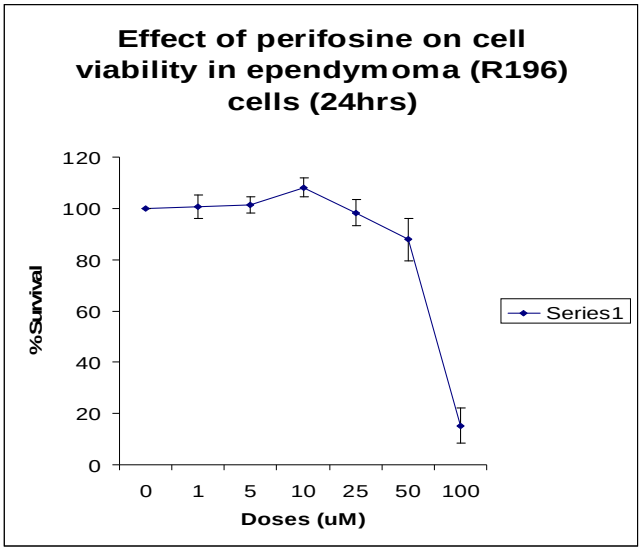


Figure C1

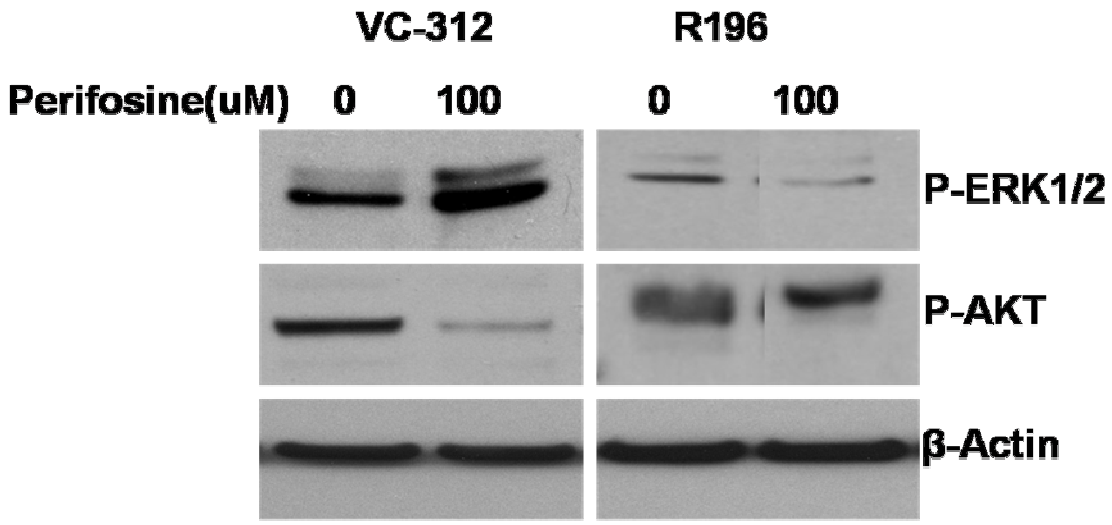


Figure C2

APPENDIX D

Combination treatment of perifosine with Akti IV has an additive effect on cell viability in medulloblastoma cells.

Rationale

We showed that perifosine potently inhibits Akt kinase and leads to a decrease in cell viability in medulloblastoma. Similar to perifosine, Akti IV is another potent Akt inhibitor, which decreases the cell viability in these cells. The mechanism of action of Akti IV is different than perifosine as it inhibits PDK1 kinase (Calbiochem/EMD Bioscience). In this study, we determined the combined effect of perifosine and Akti IV on cell viability in medulloblastoma cells.

Methods

Cells were plated in white, opaque-walled, sterile, 96-well plates at the density of 1000 cells per 100 µL of growth medium per well. After 24 hours, these cells were treated with increasing doses of perifosine (10µM to 30µM) versus vehicle control (PBS) for 24 hours. After 24 hours of treatment with perifosine, these cells were then treated with Akti IV (Calbiochem) (0.1uM and 0.3uM) for another 24 hours. Cell viability was determined using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI). The cell viability was calculated with the following equation: % Survival = (x/y)*100 where 'x' is the number of viable cells in conditions exposed to perifosine, 'y' is number of cells treated with vehicle control

Results and discussion

Treatment with combination of perifosine (10uM) and AktiIV (0.1uM) has more than additive effect on cell viability in DAOY cells. There was a 10% decrease in cell viability at 10uM perifosine and a 5% decrease in cell viability at 0.1uM AktiIV. The combined treatment of AktiIV (0.1uM) and perifosine (10uM) resulted in 35% decrease in cell viability ($P < 0.05$). N=1: The experiment was done with five replicates for each condition. On x axis labeled A is AKTi IV and P is perifosine

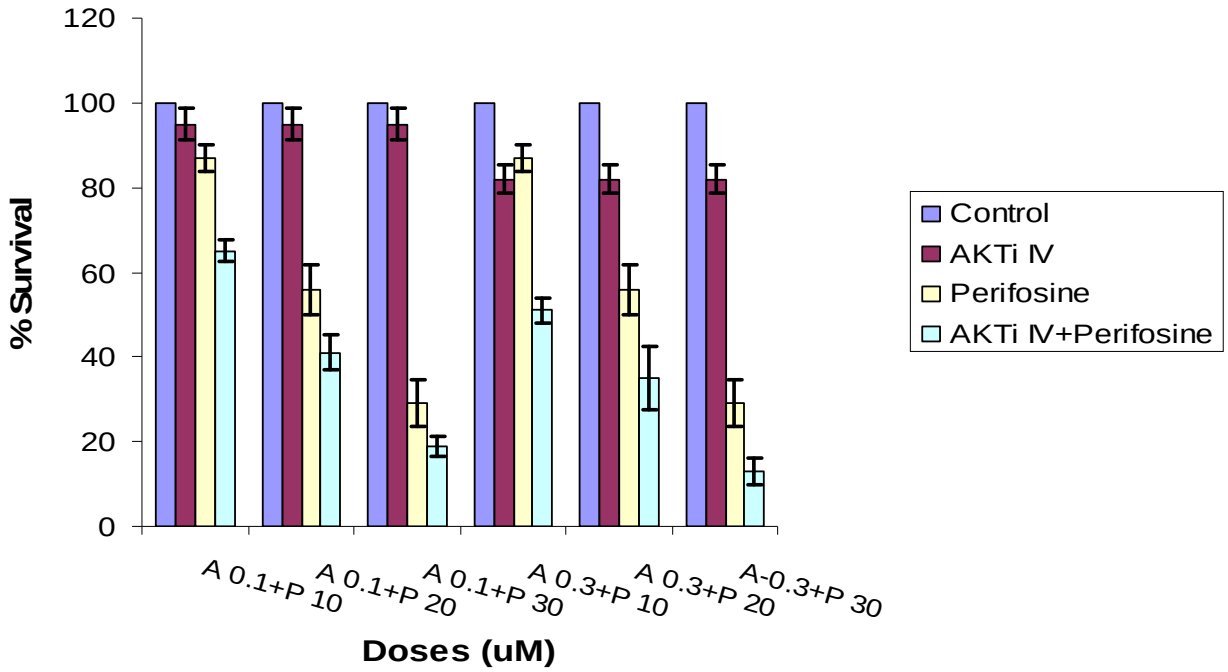


Figure D

VITA

Anil Kumar was born on October 10, 1976 in Mirchpur, Haryana, India. Anil is currently a citizen of India. He grew up in Jind and passed high school from JNV Khunga Kothi, Jind, Haryana, India. He went to medical school at the Pandit Bhagwat Dayal Sharma Institute of Medical Education and Research (PGIMS), Rohtak, India and graduated with an M.B.B.S. degree in 2001. After finishing medical school, Anil worked as physician in the Department of Cardiology at PGIMS Rohtak for one year. He then joined the Department of Anatomy as a teaching and research assistant at PGIMS, Rohtak for two years. He pursued his PhD in the Department of Anatomy and Neurobiology at Virginia Commonwealth University in Richmond, Virginia beginning in 2004. During his doctoral work he served as teaching assistant for the Dental gross anatomy and histology course. He completed his doctoral work in Anatomy and Neurobiology at Virginia Commonwealth University, Richmond, Virginia in May, 2010. Anil will be starting a residency in Neurology at Hahnemann University Hospital-Philadelphia, PA, starting July 2010 and is currently doing internship in internal medicine at Mercy Catholic Medical Center, Philadelphia.

He has been recognized for his outstanding academic performance during medical school. Anil received a scholarship for his outstanding academic grades in Anatomy. He was awarded a travel award of $2000 from Keryx Biopharmaceuticals for Poster presentation at: AACR-NCI-EORTC International Conference Molecular Targets & Cancer Therapeutics (2007, October), San Francisco, CA. His first manuscript; 'The alkylphospholipid perifosine induces apoptosis and $p21^{waf1/cip1}$-mediated cell cycle arrest in medulloblastoma' has been accepted for publication by Molecular Cancer Research.