I, Swagata Karkare, hereby submit this original work as part of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

It is entitled:
Direct inhibition of Retinoblastoma phosphorylation by Nimbolide causes cell cycle arrest and suppresses Glioblastoma growth

Student’s name: Swagata Karkare

This work and its defense approved by:

Committee chair: Giovanni Pauletti, Ph.D.

Committee member: Biplab Dasgupta, Ph.D.

Committee member: Gerald Kasting, Ph.D.
ABSTRACT

Glioblastoma multiforme (GBM) is the most common and, simultaneously, most aggressive form of primary brain tumor occurring in human adults. Despite clinical application of various chemotherapy regimens, radiation, and surgical approaches, the median survival of patients after GBM diagnosis does not exceed 15 months. This underscores the urgent need for development of new synthetic or naturally-derived therapeutic agents, which not only extend median survival beyond 15 months but also offer the potential for cure. Recent advances in delineating the contributions of distinct signaling pathways and genetic alterations in tumorigenesis offer new therapeutic targets for pharmacological interventions in GBM patients. For centuries, medicinal plants were used as essential sources for the discovery of new anticancer agents. However, to rise to the level of clinically valuable adjuvants in cancer patients, it is imperative to understand the mechanisms of action associated with plant extracts or individual components purified from medicinal plants. Using contemporary molecular biology techniques, the results from this study demonstrate that pharmacologically active ingredients present in the ethanol-soluble fraction of *Azadirachta Indica (Neem)* leaves (Azt), including nimbolide, induce significant cytotoxicity against GBM cells *in vitro* and *in vivo*. Azt caused cell cycle arrest, most prominently at the G1-S border in GBM cells expressing the EGFRvIII oncogene, which is present in about 20-25% of
glioblastomas. Azt directly inhibited kinase activity of the cyclin-dependent kinases CDK4/CDK6 leading to hypophosphorylation of the retinoblastoma (RB) protein and cell cycle arrest at G1-S. Independent of RB hypophosphorylation, Azt also significantly reduced proliferative and survival advantage of GBM cells by downregulating Bcl2 and blocking growth factor-induced phosphorylation of Akt, Erk1/2 and STAT3, respectively. In contrast, mTOR and other cell cycle regulators were not affected by Azt. Following intratumoral injection of Azt using a subcutaneous mouse GBM xenograft model, initiation and glioblastoma growth were significantly reduced as compared to ethanol treated controls. Taken together, our findings demonstrate that Azt and nimbolide effectively suppress growth of glioma cells in vitro and in vivo through interference with vital cell cycle regulators such as RB. These findings suggest that nimbolide may offer substantial clinical benefit for GBM patients and, thereby, holds incredible promises for future clinical studies.
I hereby disclose that there is no conflict of interest with regards to the following document.
ACKNOWLEDGEMENTS

“Tell me and I forget, teach me and I may remember, involve me and I learn.”

— Benjamin Franklin

I would like to express my gratitude and appreciation for the committee chair, Dr. Giovanni Pauletti. He gave me an opportunity to pursue my Master’s degree at the University of Cincinnati and allowed me to work on an independent cancer biology project in the laboratory of Dr. Biplab Dasgupta at Cincinnati Children’s Hospital Medical Center. Dr. Pauletti has perpetually conveyed a spirit of scientific & conversant attitude towards research and scholarship to all of his students. I am also immensely thankful to Dr. Gerald Kasting for his compassion, support and thoughtful guidance and for his involvement in the progress of my degree and future endeavors.

I consider myself extremely lucky to have had the good fortune of meeting an outstanding advisor like Dr. Biplab Dasgupta, who presented me with a chance to work on an independent project during my time as a graduate research assistant in his lab. As Dan Rather said, the dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, sometimes poking you with a sharp stick called "truth." Lab members, Dr. Rishiraj Chhipa, Dr. Sarah Potter and Xiaona Liu, all of whom I consider as mentors, helped me in numerous ways and I...
earnestly express my gratitude to them for training and supervising my lab-skills with an experienced & open approach to science.

Lastly, I would like to thank my friends and family for these two remarkable years of endless backing and marvelous times which brought me to the exhilarating pedestal I am at today!
# TABLE OF CONTENTS

1. INTRODUCTION........................................................................................................Page Numbers

   Background................................................................................................................12-33

2. RESEARCH OBJECTIVES.......................................................................................34

   2.1. Hypothesis

   2.2. Specific Aims

3. EXPERIMENTAL TECHNIQUES..............................................................................35-39

   3.1. Preparation of Ethanolic Neem leaf Extract (Azt)

   3.2. Cell Culture

   3.3. Cell Cycle Analysis

   3.4. Anchorage independent growth

   3.5. Western blot analysis

   3.6. Nonradioactive in vitro kinase assay

   3.7. In vivo Experiments

   3.8. Immunohistochemistry

   3.9. Statistical analysis
4. RESULTS .............................................................................40-58

4.1. Cytotoxicity of Azt and nimbolide against GBM cells in vitro

4.2. Mechanism of action of Azt/nimbolide in EGFRvIII expressing GBM cells

4.3. Effect of Azt/nimbolide on anchorage-independent growth of GBM cells

4.4. Effect of Azt/nimbolide on cell signaling cascades regulating cell cycle progression

4.5. Reversibility of Azt/nimbolide-induced cytotoxicity

4.6. Effect of Azt/nimbolide on GBM tumor growth in vivo

5. DISCUSSION.................................................................59-62

6. CONCLUSION.................................................................63-67

Future work

7. BIBLIOGRAPHY.................................................................68-81
LIST OF FIGURES

Fig 1: Classification of Glioma tumors.........................12

Fig 2: Classification of GBM.................................14

Fig 3: Signaling pathways altered in GBM.........................18

Fig 4: Role of RB in cell cycle / tumor progression..................22

Fig 5: Chemical constituents of Azadirachta indica .................29

Fig 6: External morphology of neem leaves.........................30

Fig 7: Chemical structure of nimbolide..........................31

Fig 8: Azt/ nimbolide is a potent cytotoxic agent in Glioblastoma........42

Fig 9: Azt/nimbolide arrests GBM cells at G1-S..................45

Fig 10: Azt/nimbolide inhibits anchorage-independent growth of GBM cells........47

Fig 11: Azt/nimbolide induces apoptosis, downregulates Bcl2, inhibits Akt and CDK4/6 kinase activity.........................50

Fig 12: Cytotoxicity of Azt/nimbolide is irreversible...............53

Fig 13: Azt/nimbolide suppresses tumor growth in vivo.............57
LIST OF TABLES

Table 1. Most common genetic alterations in GBM……………………..17
LIST OF ABBREVIATIONS

EtOH – Ethanol (95% v/v)

GBM - Glioblastoma multiforme

RB- Retinoblastoma protein

EGFR – Epidermal growth factor receptor

Azt- Ethanolic neem leaf extract

CDK- Cyclin dependent kinase

MAPK – Mitogen activated protein kinase

PTEN- Phosphatase tensin homology

DMEM- Dulbecco’s Modified Eagle’s Medium

NF-1 - Neurofibromatosis 1
1. INTRODUCTION

PATHOBIOLOGY OF GLIOMAS

Occupational exposure to organic solvents and/or pesticides has been known as a predisposing risk factor for glioma development. Specifically in Glioblastoma multiform (GBM), presence of cytomegalovirus RNA has prompted that this infection might lead to progression of these tumors. Gliomas exhibit peak incidences in two age group specific windows, one from 0-8 years and the other in 50-70 years of age. Also, it is predominantly seen in males.

![Fig 1: Classification of Glioma tumors](image)

Classification based on Morphology

- Oligodendroglioma
- Ependymoma
- Astrocytoma

Classification based on Histology

- Grade I - More common in Children
- Grade II - Hypercellularity
- Grade III - Mitotic figures and nuclear atypia
- Grade IV - Evidence of angiogenesis and/or necrosis
GLIOBLASTOMA MULTIFORME (GBM)

Glioblastoma Multiforme (GBM) or astrocytoma Grade IV are tumors that generally occur in the cerebral hemispheres of the brain but can be found anywhere in the brain or spinal cord. They arise in the astrocytes, which are star shaped cells comprising of the supportive tissue of the brain. It is one of the most common and aggressive primary brain tumors in the adult human population with a median survival time of 12 months from diagnosis (2).

Typically, GBM is a cellular tumor with poor differentiation of cells. The cells show an increased capacity for multiplication and their morphology changes to round or pleomorphic. Sometimes the cells become multinucleated leading to nuclear atypia. It accounts for 52% of all functional tissue brain tumor cases and 20% of all intracranial tumors. According to the modified WHO classification, it is histologically similar to anaplastic astrocytoma with the exception of additional presence of necrosis.
Fig 2. Classification of GBM

Although primary and secondary tumors look similar histologically, they differ in terms of signaling pathways altered, age groups affected and clinical progression rate. The primary tumors are found in older patients, generally >60 years of age and there is no pre-existing glioma. They account for 90% of all tumors while the secondary GBM’s account for only 10% of all tumors.
Secondary GBM’s arise from lower grade (Grade II or III astrocytomas) or mixed gliomas like oligoastrocytoma.

Despite substantial progress in patient outcome in other types of human cancers and considerable progress in our understanding of the genetics and pathology of GBM, decades of therapy have failed to drastically change survival rates. At present, treatment is curative.

In general, standard treatment comprises:

- Maximal surgical resection, radiotherapy and concomitant and adjuvant chemotherapy with Temozolomide (2,3)

- Patients older than 70 years: Less aggressive therapy is sometimes considered, using radiation or temozolomide alone (4,5,6)

Primary challenges to this kind of therapeutic regimen include:

1) The resistance of tumor cells to conventional therapies- A good example of this is resistance of GBM’s to alkylating agents. MGMT removes alkyl groups from the o6 position of guanine in DNA and this protects cells against carcinogenesis induced by these agents.

2) Brain susceptibility to damage from these conventional therapies

3) Limited capacity of the brain for auto-repair
4) The presence of the blood brain barrier (BBB)- The BBB restricts entry of large molecular sized therapies and thereby prevents them from showing any therapeutic efficacy.
GENETIC & SIGNALING PATHWAY ALTERATIONS IN GBM

The most common genetic alteration seen in GBM is loss of heterozygosity on chromosome 10. It occurs in almost 60-80% of cases. In many cases the entire copy of chromosome 10 is lost while in some, different loci are deleted, thereby implicating that tumor suppressor genes are present on these loci. Although loss of gene functions occurs more frequently, there are also incidences of gene overexpression.
<table>
<thead>
<tr>
<th>Genetic alteration</th>
<th>Normal gene function</th>
<th>Incidence</th>
</tr>
</thead>
</table>
| EGFR amplification (aneuploidy of chromosome 7)/gain-of-function mutation (in frame deletion of exons 2–7 on chromosome 7) | - Promotes cell proliferation  
- Invasion, and angiogenesis  
- Induces resistance to apoptosis  
- May mediate radiation resistance | ▪ Primary GBM (36%–60%)  
▪ Secondary GBM (8%)  
▪ Anaplastic Astrocytoma’s (15%) |
| PTEN (10q23) deletion due to LOH of chromosome 10q or mutation, or methylation of PTEN promoter | - Tumor suppressor; negatively regulates Akt signaling  
- Inhibits angiogenesis  
- Poor prognosis if mutation present | ▪ LOH:  
  o primary GBM (47%–70%)  
  o secondary GBM (54%–63%)  
▪ Mutation: primary GBM (14%–47%)  
▪ PTEN promoter methylation: Primary GBM (9%) |
| PDGFR-α and -β (4q12) amplification                                             | - Stimulates cell proliferation and migration due to an autocrine loop  
- Overexpression of PDGF-B can initiate gliomagenesis when expressed in the neural stem and progenitor cell | ▪ Primary GBM (20%–29%)  
▪ Secondary GBM (60%) |
| p53 mutation (chromosome 17p)                                                     | - Cell-cycle arrest                                                                   | ▪ Primary GBM (28%)  
▪ Secondary GBM (65%) |
| RB deletion or mutation (chromosome 13q14)                                        | - Regulates cell-cycle progression                                                   | ▪ Secondary GBM (40%) |
| MDM2 amplification or mutation                                                   | - P53 regulator                                                                     | 10%–15%                    |
| MGMT promoter methylation(chromosome 10q)                                        | - DNA-repair enzyme                                                                 | ▪ Primary GBM (36%)  
▪ Secondary GBM (75%) |

**Table 1. Most common genetic alterations in GBM (85)**
ROLE OF RETINOBLASTOMA PROTEIN (RB) & OTHER PROTEINS

IN CANCER PROGRESSION

Fig 3. Signaling pathways altered in GBM (85)
**Growth factor tyrosine kinase receptor pathway**

The most frequent gain of function event in primary GBM (rarely seen in secondary GBM) is amplification of the epidermal growth factor receptor (EGFR). It is often associated with a ligand-independent, constitutively active genomic deletion variant called EGFRvIII (also known as de2-7EGFR or pEGFR), which is most frequently overexpressed in GBM’s, sometimes by as much as 60% (7, 8, 10, 11). The EGFRvIII mutant contains an in-frame deletion of amino acids 6 to 273, thus forming an oncoprotein.

Studies indicate that tumors expressing EGFRvIII are linked to radiation and chemotherapeutic resistance (as in the case of cisplatin resistance due to a Bcl-XL modulation) and these cancers exhibit a more aggressive disease and poorer prognosis (13, 14). In addition, EGFRvIII is more tumorigenic than the wild-type receptor, which might be due to its inability to be downregulated by endocytosis (15). EGFR as well as its ligands are known to have various roles in cell proliferation, differentiation, motility, survival and tissue development (9). It has been observed that only 10 to 20% of patients have a response to EGFR kinase inhibitors. In one *in vitro* study, GBM cells were found to be sensitive to erlotinib (a kinase inhibitor) upon EGFRvIII and PTEN coexpression (12). Furthermore, Mellinghoff proved that coexpression of EGFRvIII and PTEN was significantly associated with clinical response to EGFR kinase inhibitors in patients treated with them. This frequent expression of EGFRvIII, specifically
in tumors combined with low expression in normal tissues makes it an ideal therapeutic target for kinase inhibitors, immunotoxins, and peptide vaccines.

**Ras signaling**

Specific mutations affecting this pathway are not clearly known but it is believed that this pathway becomes active by receptor tyrosine kinase activation or by the loss of function of a protein product of the large neurofibromatosis 1 (NF1) gene, neurofibromin. Ras-GTP is converted to Ras-GDP by the negative regulation of NF1. Studies indicate that a combination of Ras and Akt can induce tumors in animal models although neither of them can do so alone (86). There have also been reports of GBM-like tumors in mice models deficient in NF-1 and p53 (87).

**TGF-β signaling**

TGF-β acts as a tumor suppressor gene in GBM by downregulating CDK’s by activating its inhibitors p15, p27 and cip/WAF1/p21 (88, 89). TGF-β activates other mitogenic pathways of GBM such as MAPK which lead to proliferation and tumorigenesis.

**P53-MDM2-p14ARF pathway**

ARF (stands for alternate reading frame locus) acts as a tumor suppressor by inhibiting ribosome biogenesis or initiating p53-dependent cell cycle arrest and apoptosis, respectively (22). ARF is induced in response to sustained mitogenic stimulation, such as aberrant growth signaling from Myc & Ras
proteins (19). It is located near the genes for the tandem repeats INK4α and INK4β, which are 16 kDa (p16\textsuperscript{INK4α}) and 15 kDa (p15\textsuperscript{INK4β}) proteins, respectively. ARF or p53 play a direct role in inhibiting CDK4/6, so if these proteins were to be blocked, the tumors would be able to survive by uninhibited proliferation (18). Secondary GBM’s have >65% incidences of p53 mutations while up to 76% cases of primary GBM show loss of p14\textsuperscript{ARF} (90, 91)

**Retinoblastoma Pathway**

When RB is phosphorylated by cyclin D and E-dependent kinases in the late gap 1 phase (G1 phase), it becomes inactive and cannot block the elongation factor E2F which is responsible for transcription. Due to this, the cell progresses to the DNA synthetic phase (S phase) (20, 23, 25, 26). Therefore, INK4α and INK4β serve as tumor suppressors by restricting proliferation through the inhibition of the CDKs responsible for RB phosphorylation (21).
Fig 4. Role of RB in cell cycle / tumor progression

In the M-G1 phase transition, protein phosphatase-1 activates pRB by dephosphorylating it. RB then acts as a tumor suppressor by inhibiting cell cycle progression (23, 24). RB remains phosphorylated throughout S, G2 and M phases (23). Free E2F activates factors like Cyclin E and A which activate CDKs and cause cell cycle progression. It also activates the proliferating cell
nuclear antigen (PCNA), which attaches polymerase to DNA and thus is responsible for DNA repair and replication (25, 27, and 28).

**PTEN/Akt-1 pathway**

Two tumor suppressors frequently lost in GBM are *INK4a/ARF* that regulates the retinoblastoma (*RB*) protein and phosphatase tensin homology (*PTEN*) that regulates *PI3K* (16, 17). Growth factor pathways often upregulated in GBMs includes the PI3K-Akt, MEK-Erk1/2 and the JAK-STAT3 pathways (29). Akt (or protein kinase B) plays a significant role in cancer progression and tumors with an activated PTEN/Akt pathway may be sensitive to mTOR inhibitors like rapamycin (82). Tyrosine kinases are activated by coupling of the 85-kD adaptor subunit of PI3K to its 110-kD subunit. Tyrosine kinases in turn activates the Akt cascade by utilizing phosphoinositide 3-kinase (PI3K) to produce phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5)P3). It is believed that PTEN acts a tumor suppressor by antagonizing protein tyrosine kinases (which are involved in cell metastasis and invasion) (30).

PTEN suppresses GBM by blocking cell cycle progression in the G1 phase. Studies suggest that the p27 is an important target when PTEN acts as a tumor suppressor by negative regulation of the PI3K/Akt signaling pathway (32, 83, and 84). In addition, Weng demonstrated that PTEN causes cell growth suppression by inhibiting Akt, upregulating p27, downregulating cyclin D1 and thus blocking the cell cycle progression (33).
Akt regulates cell growth through its effects on the TSC1/TSC2 complex and mTOR pathways. In addition, it directly acts on the CDK inhibitors p21 and p27 to regulate cell cycle and cell progression. Akt inhibits pro-apoptotic Bad and Fox0/Myc transcription factors to mediate cell survival (34, 35). Amongst the different signal transduction pathways, the mitogen-activated protein kinase (MAPK) pathway plays a fundamental role in that it brings together a number of different extracellular signals influencing a variety of phenomena involved in cellular behavior such as proliferation, migration, differentiation or survival (36). This pathway is characterized by the activation of Ras GTPase, leading to an activation of the serine/threonine kinase Raf which in turn phosphorylates MEK (MAP/ERK kinase), the latter activating extracellular signal-regulated kinases (ERks). In cancer research the MAPK cascade leading to ERK1/2 activation has been subject of particular interest as this pathway is activated in a broad spectrum of tumors and may thus represent a potential molecular target for therapeutic intervention (37). The cellular outcome of ERK1/2 signaling is complex, because signal specificity can be modified by the duration, magnitude and subcellular localization of ERK activation, thereby even leading to opposite effects, i.e. cell proliferation versus cell cycle arrest and cell differentiation. Paternot and Roger (2009) have demonstrated that combined perturbation of MAPK/ERK1/2 and PI3/Akt/mTOR signaling prevents proliferation of glioma cells more efficiently than inhibition of a single pathway (38).
MULTI-TARGETED THERAPY

Attempts to pharmacologically inhibit individual pathways dysregulated in GBM for effective therapy have failed. As opposed to targeted therapy, promiscuous drugs that inhibit multiple pathways are becoming a virtue in drug development as in the case of sorafenib (targets growth factors and is in phase II clinical trials for GBM), dasatinib (targets cytoplasmic kinases especially PI3K/mTOR and is in phase II trials for recurrent GBM) and cilengitide (targets cell cycle integrins and is in Phase I trial for pediatric brain tumors and gliomas). This is the reason for increasing design of therapies to target multiple kinase pathways (39). It can be achieved using a single agent that inhibits multiple signaling pathways or a combination of highly selective agents. Disadvantages of this type of therapeutic approach might include possible increased cost and toxicity. In addition, differing affinities for the receptors may result in relatively greater inhibition of one target in comparison to another resulting in toxicity. In contrast, combining selective agents with the aim of achieving additive or synergistic effects may allow high target selectivity with reduced systemic effects, though this is at the risk of potential pharmacodynamic and pharmacokinetic interactions between the drugs. Ideally, combination therapies should use effective agents with differing mechanisms of action and adverse effect profiles. Chemical compounds occurring in plants or phytochemicals, used as single defined chemical entitites or a diverse array of
these chemicals with limited characterization using techniques like fingerprint analysis, are time-tested for their curative properties against a plethora of chronic human diseases. Because of their general safety, long term use and the possibility to target multiple pathways, there is a renewed interest to understand their molecular mechanisms of action. For example, dietary phenolic compounds and isothiocyanates isolated from the edible vegetables of the genus *Brassica* induce cell cycle arrest by stabilizing p21 and p53 (40, 41), while curcumin and resveratrol (both in cancer clinical trials) induce apoptosis by downregulating Bcl2 and upregulating Bax, thus making them favorable chemotherapeutic agents (41, 42). Organosulphur derivatives from garlic exert anticancer effects by downregulating NF-κB (43). Recently, trabectedin (brand name Yondelis), a natural product of obtained from the sea squirt *Ecteinascidia turbinata* (also in clinical trial for the treatment of breast, prostate and pediatric sarcomas and approved for treatment of soft tissue sarcoma) induced apoptosis specifically in tumor macrophages (44).
**AZADIRACHTA INDICA / NEEM**

*Azadirachta indica* (A.juss) Family *Meliaceae* is a medicinal plant with a long history of use for over 2000 years in primary health care in South east Asia (45). It belongs to the class *Magnoliopsida* or dicotyledons and the subclass *Rosidae*. Numerous bioactive phytochemicals with medicinal properties have been isolated from various parts of this plant (46). Fractionated extract of its leaves, known as IRAB is registered and marketed in Africa as IRACARP® for its broad-spectrum anti-cytoadhesion activity and its beneficial effects in HIV/AIDS (47, 48).

*Azadirachta indica* (popular as Neem or Margosa) has been known as a rich source of limonoids that have potent antioxidant and anti-cancer properties. In India, it is popularly known as the ‘sacred tree’ or ‘nature’s drugstore’ or ‘village pharmacy’. It is considered a major component of Ayurvedic and Unani medicines and is particularly prescribed for skin disease (49). In Farsi, *Azad* means free, *Dirakht* means tree and *i-Hind* means of Indian origin.
**VARIOUS USES OF NEEM**

- **Antimalarial effects:** Gedunin has been reported as the antimalarial agent of *Azadirachta indica* (55). Azadirachtin and its semi-synthetic derivatives block the *in vitro* development of motile male malarial gamete (56).

- **Antimicrobial effect:** NIM-76, obtained from neem oil has spermicidal properties and causes viral inactivation (57).

- **Contraceptive effects:** It is hypothesized that neem constituents may be absorbed and transferred to susceptible organs like the ovaries or phagocytic cells may uptake them. Literature reports abortion preceded by a decrease in progesterone and chorionic gonadotropin (CG) in monkeys leading to a failure in maintenance of the endometrium and pregnancy (58). According to another study, however, neem did not injure corpus luteum function in nonpregnant female baboons (59).

- **Pesticidal effects:** Azadiractin has shown to interrupt metamorphosis in insects, causing pesticidal effects (60).

- **Toxicological effects:** Neem oil (obtained from fruits or seeds) causes mitochondrial dysfunction and energy crisis. Various mechanisms for this might be uncoupling of mitochondrial oxidative phosphorylation, decreased ATP and inhibition of the electron-transport chain (61). According to one report, neem oil caused mitochondrial swelling in rat liver mitochondrial models (62).
**Fig 5. Chemical constituents of *Azadirachta indica***

**Constituents of neem bark**

Neem barks contain nimbin, nimbinin, nimbidin and nimbindiol. Neem barks and leaves also contain tannins and non-tannins (oils).

**Constituents of neem flowers**

Quercetin and nimbosterol are present in the neem flowers.

**Constituents of neem seeds**

Active constituents have not been determined with certainty (52). Azadirachtin H [3] and azadirachtin I [4], which are tetranoctriterpenoids have been isolated.
from neem seeds (53, 54). In addition, salanin and meliacin are also present in the neem seeds.

**Constituents of neem Leaves**

The leaves of Neem are opposite, pinnate, 7.9 to 16 inches long with 20-31 medium to dark green leaflets about 1.2-3.1 inches long. The terminal leaflet is often missing.

![Fig 6. External morphology of neem leaves](image)

- **Constituents: Leaf extracts:**

  Active constituents of neem leaf extract include isomeldenin, nimbin, nimbinene, 6-desacetylnimbinene, nimbandiol, nimbolide, nimcocinol (50, 51).
Methanolic extracts of the leaves yielded tetracyclic triterpenoids, zafaral and meliacinanhydride (50).
The pharmacologically active cytotoxic component of the leaf extract is called nimbolide, a tetranortriterpenoid consisting of a classical limonoid skeleton with an $\alpha,\beta$-unsaturated ketone system and a $\delta$-lactonic ring (48). The ethanol-soluble fraction of *Azadirachta indica* leaves (henceforth called Azt) as well as nimbolide have been shown to exert several biological activities including anti-malarial (64), anti-HIV (48) anti-cancer response (65) and anti-satiety response (63). A few studies have explored molecular mechanism of action of nimbolide and it was demonstrated *in vitro* that it sensitizes colon cancer cells to TRAIL induced apoptosis through three distinct mechanisms: ERK-mediated up-regulation of DR5 and DR4, downregulation of cell survival proteins, and up-regulation of pro-apoptotic proteins p53 and Bax (31, 66). In a recent study, Kumar and co-workers investigated the cytotoxic effects of nimbolide on
human choriocarcinoma (BeWo) cells. Nimbolide showed an inhibition of proliferation and induction of apoptosis in these cells in a dose- and time-dependent manner by down regulating proliferating cell nuclear antigen, increasing reactive oxygen species production and decreasing Bcl-2/Bax ratio. Results of the study suggested that nimbolide has immense potential in cancer prevention and therapy (67).

**PROPERTIES OF NIMBOLIDE** (adapted from Biovision)

It is a white to off-white solid powder with the following toxicity profile.

**Nimbolide Toxicity Profile:**

Acute toxicity: LD50 Oral/Intraperitoneal – rat – >600 mg/kg

LD50 Oral – mouse – >600 mg/kg

LD50 Intraperitoneal – mouse – 225 mg/kg

LD50 Intravenous – mouse – 24 mg/kg

Azt/nimbolide exhibits anti-cancer properties against a variety of tumor cells including neuroblastoma, osteosarcoma, leukemia and melanoma cells (66-69). These cancer cells are variously affected, likely due to the interaction of Azt with the unique pathways mutated in these cells. Some of the pathways involved in Azt action include cell cycle arrest at G0/G1 (69), increased ROS production (67), activation of caspases, modulation of the levels of cell cycle inhibitors and suppression of NF-κB activity (66). In animal tumor models,
nimbolide (10–100 mg/kg) has been shown to exhibit chemopreventive activity against 7,12-dimethylbenz[α]anthracene-induced oral carcinogenesis (65, 70). The αβ – unsaturated ketone structure of nimbolide is linked to its anti-cancer property (71) while amide derivatives modified on the lactone ring enhanced its cytotoxicity (72).
2. RESEARCH OBJECTIVES

2.1. HYPOTHESIS

Ethanolic neem leaf extract (Azt) and the principal cytotoxic component of the extract, Nimbolide, inhibits CDK4/6 activity to cause cell cycle arrest, apoptosis and suppression of glioblastoma growth \textit{in vitro and in vivo}.

2.2. SPECIFIC AIMS

1) To examine the \textit{in vitro} cytotoxic potential of Azt and nimbolide against glioblastoma cell lines.

2) To determine the effect of Azt and nimbolide on tumor progression \textit{in vivo} using tumor xenografts of U87EGFRvIII cells in athymic mice models.
3. EXPERIMENTAL TECHNIQUES

3.1. Preparation of Ethanolic Neem Leaf Extract

Dried powder of *Azadirachta indica* leaves was provided by Dr. Ghosh. He prepared it by drying fresh leaves at 37°C for 24h and grinding them into a powder using a regular grinder. 1 gram of powder was macerated in 5ml EtOH (95%v/v) and Azt was extracted at 4°C on a shaker for five days. Insoluble solutes were precipitated by centrifugation and the supernatant was filtered through a 0.2 micron filter. Azt was stored in aliquots at -20°C until further use.

3.2. Cell Culture

Glioblastoma cell lines T98G, A172 and U87 were purchased from American Type Culture Collection (Manassas, VA, USA). G2 cells were obtained from Dr. Peter Houghton, Nationwide Children’s Hospital, Columbus, OH. U87EGFR and U87EGFRvIII cells were provided by Dr. Paul Mischel, UCLA and were maintained in puromycin (1µg/ml) and hygromycin (150 µg/ml), respectively. The primary adult GBM neurosphere cell line, P9 was obtained from Dr. John Ohlfest, University of Minnesota. All glioma cells except P9 were cultured in standard cell culture treated petridishes in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (100 µg /ml penicillin G, 100 µg/ml streptomycin) at 37°C in 5% CO₂ and 95% air in a humidified incubator. Upon reaching confluency, the cells were trypsinized and passaged. P9 cells were cultured in ultra-low attachment
dishes in DMEM/F12 supplemented with B27, N-2 supplement, EGF and FGF. Cell viability was determined by trypan blue exclusion method.

### 3.3. Cell Cycle Analysis

Cell cycle distribution was performed by flow cytometry. 2.5 x 10^5 Cells were treated with EtOH (control) or Azt (2 μg/μl for 12 hour and 1 μg/μl for 24 hour), harvested, fixed with 70 % ice cold EtOH at -20 °C for 1 h and resuspended in 0.5ml of propidium iodide/ RNase staining buffer. Cells were filtered through a 70μm Sefar Nylon Lab Pak Mesh. DNA content was analyzed on a Beckman Coulter Quanta™ SC MPL Flow Cytometer.

### 3.4. Anchorage independent growth

For Anchorage independent growth, 2 x 10^4 GBM cells were mixed with 0.7% top agar and layered on top of 1% bottom agar made in 2X DMEM with 20% fetal calf serum and penicillin/streptomycin. Cells were maintained with medium containing EtOH, DMSO (control) or Azt, nimboide purchased from BioVision (Milpitas, CA, USA) every third day and allowed to grow for two weeks. Colonies were stained with crystal violet and imaged with a Nikon AZ-100 multi-zoom microscope attached with DS-RI1 12 mp color camera. Colony quantitation was done using Image J software.
3.5. Western blot analysis

Glioma cells were lysed with RIPA lysis buffer (20mM Tris, 10 mM EGTA, 40mM β-glycerophosphate, 1% NP40, 2.5mM MgCl$_2$, 2mM orthovanadate 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail) and protein concentrations were determined by the BCA assay method. The following antibodies (all from Cell Signaling Technology) were used: Cleaved PARP, Bcl2, pRB, pAkt, Akt, pGSK3β, GSK3αβ, pCyclinD1, Cyclin D1, pErk1/2, Erk1/2, pSTAT3, STAT3, pS6, S6, p4EBP1, 4EBP1, Cyclin B1, P27, P21 and actin. The proteins were electrophoresed through an SDS-polyacrylamide gel and transferred onto PVDF membranes according to standard protocols. Overnight incubation at 4°C in primary antibodies was followed by incubation with appropriate horseradish peroxidase–conjugated secondary mouse or rabbit antibodies. Peroxide activity was detected using the enhanced chemiluminescence.

3.6. Nonradioactive in vitro kinase assay

Nonradioactive in vitro kinase assay was performed by examining phosphorylation on a GST-RB fusion peptide (residues 736–840). GST-RB1 construct containing residues 736-840 of RB purchased from Addgene (Cambridge, MA, USA) was purified according to standard protocol. GST-RB1 was expressed in Rosetta 2 (DE3) pLys cells. Cells were lysed by sonication in 20mM Tris pH8.0, 300mM NaCl (Buffer A). Lysate was loaded on glutathione
Superflow beads (Qiagen), equilibrated with Buffer A. Following extensive wash in Buffer A, GST-RB1 was eluted with Buffer A supplemented with 50mM Glutathione. Fractions containing GST-RB1, as judged by SDS-PAGE, were pooled, dialyzed against 20mM Tris pH 8.0, 200mM NaCl, 0.1% β-mercaptoethanol and concentrated to 2mg/ml as measured by BCA assay (Amicon, 10kDa cutoff). Kinase assay was conducted as previously described (25). Briefly, U87EGFRvIII were lysed with RIPA buffer, and CDK4/6 was immunoprecipitated with Cyclin D1 antibody, washed twice with RIPA buffer, once with 10 mM Tris-Hcl (pH 7.5) and 0.5 M LiCl, once with kinase assay buffer and suspended in kinase buffer (25mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4,, 10mM MgCl2). Reactions were carried out at 30°C for 45 minutes by adding GST-RB fusion protein and 250 μM ATP to the reaction mix. Reaction was terminated by adding Laemmli buffer and boiling for 5 min. Samples were resolved in 14% SDS-PAGE, and Western blot analysis was performed with phospho-RBSer807/811 antibody (Cell Signaling Technology). Peroxide activity was detected using the enhanced chemiluminescence.

3.7. In vivo Experiments

For in vivo xenografts experiments, 1.5 x 10⁶ U87EGFRvIII cells suspended in phosphate buffered saline were injected into the flanks of nu/nu female mice (n=8). Mice were anesthetized by inhalation of isoflurane. For glioma initiation
experiment, cell were pretreated with EtOH (control) or Azt for 1h and injected into the flanks. Mice did not receive any further injections. For glioma progression experiment, similar numbers of untreated cells were injected and tumors were allowed to grow for seven days. Following this, 62.5 ul of EtOH or Azt (0.34 μg neem leaf powder/g body weight) was injected directly into the tumor bed of the left and right flank respectively, every other day for one week. Tumor growth was monitored by measuring tumor dimensions every other day by using calipers. The volume was calculated according to $\pi/6 \times A \times B^2$ ($A =$ larger diameter; $B =$ smaller diameter). Tumors were dissected out and imaged using an iPAD 3.

3.8. Immunohistochemistry

Immunohistochemistry was done as previously described (25). Mice were anesthetized by inhalation using isoflurane, perfused intracardially with 4% PBS, tumors were dissected out and processed for paraffin embedding and sectioning. Tissue sections were stained with H&E. Immunohistochemistry was performed on adjacent deparaffinized six-micron sections by using standard antigen retrieval methods. The following primary antibodies were used: Ki67 (Vector laboratories), cleaved Caspase 3, pAkt, pErk1/2, pSTAT3 (Cell Signaling Technology). Vectastain ABC kit (Vector laboratories) was used for microscopic visualization. Histology images were taken with a Leica DM2500 brightfield upright microscope attached with a DFC 500 12 MP color camera.
3.9. Statistical Analysis

Student's *t* test was used to assess statistical difference between two experimental groups (Graph Pad Prism 5.0, Graph Pad Software Inc., San Diego, CA), with $p < 0.05$ representing a statistical significance.
4. RESULTS

4.1. Cytotoxicity of Azt and nimbolide against GBM cells \textit{in vitro}

Initially, the concentration dependent cytotoxicity of an aqueous vs. ethanolic extract of \textit{Azadirachta indica} leaves was compared \textit{in vitro} using the T98G GBM cell lines. The results summarized in Fig 8A demonstrate that the aqueous extract did not negatively affect cell viability up to a concentration of 4µg of leaf powder per µl of cell culture medium. In contrast, extract prepared with EtOH killed T98G GBM cells in a dose dependent manner (Fig 8A). At 4 µg/µl, Azt killed 100% of the cells in 20 hours under DMEM/10% FCS culture conditions. In comparison, Temozolomide (TMZ), which is clinically used as an adjuvant in standard of care of GBM patients, was significantly less cytotoxic to GBM cells after a 72 hr. treatment (Fig 8B). The effective cytotoxic potency of Azt was demonstrated across various GBM cell lines, including two adult GBM cells that overexpresses EGFR or EGFRvIII, one adult primary GBM neurosphere line (P9) and one pediatric GBM line (G2) (Fig 8C). Photomicrographs of A172 and U87 EGFRvIII cells treated with Azt for 20 hours are shown in Fig 8D, E. Even at the lowest dose of Azt (1µg/µl), the shape of U87EGFRvIII cells is rounded, suggesting substantial cytotoxicity. Interestingly, parental U87 cells maintained high cell viability in the presence of low (<1µg/µl) Azt concentration. However, 50-70% U87EGFRvIII cells were killed at 0.75 and 0.875 µg/µl Azt, respectively (Fig 8F). These results suggest
that constitutively active signaling through the EGFRvIII oncogene may induce an oncogenic stress and consequently enhance vulnerability of GBM cells towards Azt. To explore whether this effect requires a complex mixture of active components in Azt, similar experiments were conducted with nimbolide (Fig 8G), which is considered the principal cytotoxic component in Azt (92). Similar to Azt, nimbolide considerably reduced cell viability of in a dose dependent manner, killing GBM cells that express the EGFRvIII oncogene more effectively (Fig 8H). Taken together, these results suggest that nimbolide may be primarily responsible for the cytotoxic effects associated with Azt against GBM cells in vitro.
Fig 8. Azt/ nimbolide is a potent cytotoxic agent in Glioblastoma
(A) Cytotoxic effect of aqueous or EtOH extracts (Azt) of *Azadirachta indica* on T98G human glioblastoma (GBM) cells (A) and that of Temozolomide (TMZ) on T98G, U87 and U87EGFRvIII cells (B). (C) Dose-dependent cytotoxicity of Azt in a panel of human GBM cells treated with Azt or ethanol (control) for 20 hours. (D, E) Photomicrographs showing morphology of GBM cells (control-EtOH treated, attached) but lifting from tissue culture plates following Azt treatment for 20h. (F) GBM cells expressing the EGFRvIII oncogene are sensitive to lower doses of Azt than parental cells. Cytotoxicity of nimbolide (G), the principal cytotoxic component of Azt on a panel of GBM cells (H). Note: U87 EGFRvIII cells are more sensitive to 10μM nimbolide (circled asterisk). Data is representative of two to four experiments. *p ≤ 0.001.
4.2. Mechanism of action of Azt/nimbolide in EGFRvIII expressing GBM cells

To explore the mechanism(s) underlying Azt’s cytotoxic efficacy against GBM cells, an expanded cell cycle analysis was conducted. Histogram analysis revealed that Azt caused substantial increase in the Sub G<sub>0</sub> population of T98G cells (Fig 9A, B) and, to some extent, in U87EGFRvIII cells (Fig 9E, F). Following exposure of parental U87 cells to Azt, Sub G0 population was undetected (Fig 9 C, D). As compared to EtOH-treated control cells, the number of T98G cells in S phase doubled after Azt treatment (Fig 9A, B). In contrast, Azt-treated parental U87 cells showed a slight increase in the population of cells in G1 and a reduction in the S phase (Fig 9 C, D). Azt caused the most dramatic effect on the U87EGFRvIII cells. After a12h incubation, most of the cells were arrested in the S phase while G1 and G2M phases were dramatically depleted (Fig 9E). After 24h, > 90% of the cells accumulated at G1, concomitant with a depletion of cells from S and G2M (Fig 9F). These results suggest that background genetic variations in GBM cells influence the nature of cell cycle arrest induced by Azt. It appears that cells with the EGFRvIII mutation are significantly more vulnerable to Azt-mediated G1 arrest.
**Fig 9. Azt/nimbolide arrests GBM cells at G1-S**

Flow cytometry using PI showing cell cycle arrest of T98G (A, B), U87 (C, D) and U87 EGFRvIII (E, F) by Azt/nimbolide at 12 and 24 hours. Note that Azt treatment causes complete depletion of U87EGFRvIII cells from G2M at 12 hours and nearly 100% G1 arrest at 24 hours. Data is representative of three independent experiments.
4.3. Effect of Azt/nimbolide on anchorage-independent growth of GBM cells

The data so far demonstrated that Azt reduces viability of GBM cells when cultured as monolayers. To examine whether Azt can also inhibit anchorage-independent growth, (which correlates with tumor aggressiveness and metastatic potential *in vivo*), soft agar colony formation assays were performed. In the presence of EtOH alone (control), cells rapidly formed large colonies after two weeks. In contrast, exposure to Azt significantly decreased colony formation of all GBM cells in a dose-dependent manner (Fig 10A-D). When Azt was included after seven days of cell growth without exposure to cytotoxic compounds, colony formation was still inhibited (data not shown). This implies that Azt also negatively impacts progression of colony growth. Similar to the results obtained with Azt, nimbolide potently inhibited colony formation in a dose-dependent manner (Fig 10E-H). Therefore it was concluded that Azt and its principal cytotoxic component nimbolide are highly potent agents that inhibit anchorage-independent growth of glioma colonies *in vitro*. 
**AZT**

A. 

T98G

<table>
<thead>
<tr>
<th>EtOH</th>
<th>Azt (1μg/μl)</th>
<th>Azt (2μg/μl)</th>
<th>Azt (4μg/μl)</th>
</tr>
</thead>
</table>

B. 

U87

C. 

U87EGFRvIII

D. Colony #

- T98G
- U87
- U87EGFRvIII

<table>
<thead>
<tr>
<th>EtOH</th>
<th>Azt (1μg/μl)</th>
<th>Azt (2μg/μl)</th>
<th>Azt (4μg/μl)</th>
</tr>
</thead>
</table>

E. 

T98G

<table>
<thead>
<tr>
<th>DMSO</th>
<th>Nimb (5μM)</th>
<th>Nimb (10μM)</th>
<th>Nimb (20μM)</th>
</tr>
</thead>
</table>

F. 

U87

G. 

U87EGFRvIII

H. Colony #

- T98G
- U87
- U87EGFRvIII

<table>
<thead>
<tr>
<th>DMSO</th>
<th>Nimb (5μM)</th>
<th>Nimb (10μM)</th>
<th>Nimb (20μM)</th>
</tr>
</thead>
</table>
**Fig 10. Azt/nimbolide inhibits anchorage-independent growth of GBM cells**

Photomicrographs showing colony formation of T98G cells (A), U87 cells (B) and U87EGFRvIII cells (C) grown in the presence of EtOH (control) or Azt for two weeks. Inset shows magnified view of the colonies. (D) Quantitation of colony counts in A-C. Similar experiments were done using various doses of nimbolide or DMSO (control). Colony formations of the same cells are shown in E-G. Quantitation of colony counts in E-G is shown in H. Inset shows magnified view of the colonies. *p ≤ 0.001.
4.4. Effect of Azt/nimbolide on cell signaling cascades regulating cell cycle progression

Since Azt treatment significantly shifted cells to the sub G₀ stage (Fig 9A, B), a characteristic feature of cell death, we used cleaved PARP3 immunoblot analysis to assess the effect of Azt on apoptosis. Apoptosis was clearly evident in glioma cells treated with Azt but not with EtOH (control). (Fig 11A). Moreover, immunoblot analysis demonstrated that Azt downregulates the antiapoptotic protein Bcl2 in a time-dependent manner (Fig 11A). On the other hand, lack of extensive conversion of LC3A/B-I to LC3A/B-II in Azt-treated cells did not support extensive autophagy as a substitute mechanism involved in Azt mediated cytotoxicity. To investigate the mechanism of G1-S arrest by Azt, we examined expression of key regulators of the G1-S phase. Growth factor withdrawal inactivates CDKs that control phosphorylation of the RB tumor suppressor protein and thus, G1-S transition. As hypothesized, serum-stimulation induced robust RB phosphorylation, which was effectively diminished following exposure to Azt. This effect was most pronounced in the U87 EGFRvIII cell line (Fig 10F). Azt also suppressed serum-stimulated Akt phosphorylation (Fig 11B). Consequently, it was anticipated that Azt-mediated Akt inhibition leads to hypophosphorylation and activation of the Akt substrate GSK3β, which subsequently induces phosphorylation of the GSK3β site on Cyclin D1. Phosphorylation of Cyclin D1 by GSK3β at Thr 286 enhances its
ubiquitination and proteasomal degradation preventing CDK4-dependent RB phosphorylation. However, despite inhibition of Akt phosphorylation by Azt, GSK3β expression and Cyclin D1 phosphorylation remained unchanged (Fig 11B). Similarly, protein levels of Cyclin B1 (a crucial G2 Cyclin) and P27, P21 (two key negative regulators of the G1-S phase) were unaffected. Therefore, it was questioned whether Azt/nimbolide directly inhibits the kinase activity of CDK4/6. To test this hypothesis, CDK4/6 was immunoprecipitated from U87EGFRvIII cells with a Cyclin D1 antibody and its activity was quantified using a nonradioactive in vitro kinase assay in the presence of EtOH or DMSO (control) and Azt or purified nimbolide. The results shown in Fig 11C demonstrate that Azt/nimbolide robustly inhibited CDK4/6 activity in a dose-dependent manner. Consequently, it is highly likely that Azt/nimbolide cause a sustained inhibition of RB phosphorylation and cell cycle arrest at G1-S by inhibiting CDK 4/6 activity. From the results of this study, it appears that glioma cells carrying the EGFRvIII oncogene are particularly susceptible to this cell cycle arrest induced by Azt/nimbolide.
Fig 11. Azt/nimbolide induces apoptosis, downregulates Bcl2, inhibits Akt and CDK4/6 kinase activity
(A) Immunoblots showing the dose-dependent effects of Azt in inducing apoptosis (note: smaller bands of PARP cleaved by active Caspases at 6 & 12h of Azt treatment), the failure to induce autophagy (note: no conversion of LC3A/B-I to LC3A/B-II) and in down regulating Bcl2. Actin was used as a loading control. (B) Immunoblots showing RB and Akt phosphorylation by serum in serum-starved GBM cells and inhibition of phosphorylation by Azt but not by EtOH (control). Phosphorylation state of Akt substrate GSK3β, GSK3β substrate, Cyclin D1 and total levels of GSK3αβ, Akt, Cyclin D1, Cyclin B1, p21 and p27 are also shown (note; Azt did not change total levels of proteins or that of G1-S cell cycle inhibitors p21 and p27). (C) *In vitro* kinase assay showing dose-dependent inhibition of CDK4/6 activity by Azt (1, 2 and 4μg/μl) and nimbolide (5, 10 and 20μM) but not by EtOH or DMSO (vehicle controls). Data is representative of two to four independent experiments.
4.5. Reversibility of Azt/nimbolide-induced cytotoxicity

Our data indicates that chronic exposure of GBM cells to Azt/nimbolide suppresses growth. To explore whether Azt-induced growth suppression is reversible, GBM cells were exposed to Azt (2μg/μl), EtOH (final concentration 1% v/v) or PBS (control) for 1 or 2 hours, respectively. Following this short-term incubation, cells were washed using standard maintenance medium, and equal numbers of live cells were reseeded in maintenance for 72 hours. While the cell numbers after EtOH and PBS treatment were comparable, nearly 100% of the Azt-treated cells failed to survive and proliferate (Fig 12A). This suggests that acute exposure of U87EGFRvIII cells to Azt/nimbolide causes irreversible changes that negatively affect growth and survival of GBM cells. To investigate the signaling pathways underlying the acute toxicity effect of Azt, various growth factor pathways were monitored by immunoblot analysis (Fig 12B). Azt strongly inhibited phosphorylation of Akt, Erk1/2, STAT3 and RB signaling pathways that are vital for proliferation and survival of GBM cells. Since the mTOR pathway was not dramatically involved as demonstrated by unchanged phosphorylation of the two bonafide downstream effectors, S6 and 4EBP1, respectively, it was concluded that Azt growth inhibition is somewhat selective (Fig 12B). To explore the relevance of these in vitro experiments for the in vivo situation, based on literature data obtained with other cytotoxic drugs, it was predicted that the tissue microenvironment would allow Azt-treated cells to
survive and form tumor masses. $1.5 \times 10^6$ viable U87EGFRvIII cells treated with Azt, EtOH or PBS as outlined above were injected into the flank of nude mice. While EtOH-treated control cells consistently formed a large tumor mass, Azt pretreated GBM cells failed to survive and grow as xenografts (Fig 12C, D). Together, our results demonstrate that Azt exerts an irreversible cytotoxic effect on GBM cells in vitro that cannot be rescued in vivo.
**Fig 12. Cytotoxicity of Azt/nimbolide is irreversible**

(A) Growth assay showing viability of U87EGFRvIII cells following pre-treatment with medium (control), EtOH (control) or Azt (2μg/μl). (B) Immunoblot analysis showing activation of growth factor pathways by serum and acute inhibition of the pathways by Azt/nimbolide. Note: phosphorylation of Akt, Erk1/2 and STAT3 and RB by serum in the presence of EtOH (control) and robust inhibition of phosphorylation in the presence of Azt. Also note that mTORC1 activation, indicated by phosphorylation of S6 and 4EBP1 is unaffected by Azt. Actin was used as a loading control. Data is representative of at least three independent experiments. (C) Representative digital photograph showing tumor growth in mice injected with EtOH-pretreated (red arrowhead) or Azt-pretreated (blue arrowhead) U87EGFRvIII cells. (D) Growth of tumor xenografts in Nu/Nu mice (n=8) initiated by U87EGFRvIII cells pretreated with EtOH or Azt. Tumor volume was measured at indicated time points and the mean tumor volume was calculated. *p ≤ 0.0002.
4.6. Effect of Azt/nimbolide on GBM tumor growth *in vivo*

The results shown so far strongly support a substantial *in vitro* growth inhibitory effect of Azt/nimbolide on GBM cells viability. To verify whether Azt can also inhibit tumor growth *in vivo*, xenograft experiments with subcutaneously injected U87EGFRvIII cells were initiated in nude mice. Tumors were allowed to grow for seven days before 62.5 μl of EtOH or Azt (corresponding to 0.34 μg dried leaf powder per gram body weight) was directly injected every other day for eight days into the tumor bed. Despite EtOH treatment, tumors continued to grow readily to an average volume of 1250 mm$^3$ after 15 days. In contrast, Azt significantly reduced proliferation of GBM tumor cells *in vivo* (Fig 13 A-C). Following three injections, the mean volume of Azt-treated tumors was 50% less than that of EtOH exposed tumors. At the end of the 15 day study, Azt-treated tumors were less than 10% of the volume of EtOH exposed control tumors (Fig 13C). Histological examination using H&E staining revealed that Azt-treated tumors had markedly reduced cellularity based on comparative density of nuclei (Fig 13D & E). Furthermore, Azt markedly reduced the number of tumor cells in different stages of cell division (Fig 13 D, E; arrow heads), while increasing tumor cells with condensed/fragmented chromatin and pycnotic nuclei, which is indicative of dead cells (Fig 13 F, G and inset; arrow heads and circle). Immunohistochemistry using Ki67 antibody showed significant reduction in proliferative index of Azt-treated tumors (Fig 13 H- J).
Azt also caused apoptosis as shown by increased immunoreactivity with cleaved Caspase 3 antibody (Fig 13K-M). EtOH exposed control tumors demonstrated activated Akt, Erk1/2 and STAT3 pathways as measured by phosphorylation status (Fig 13 N, P, and R). Most importantly, Azt markedly reduced activation of all three signaling pathways in vivo, which is consistent with the in vitro findings (Fig 13O, Q, and S). From these in vivo data, it was concluded that Azt effectively inhibits multiple growth factor pathways, thereby reducing tumor cell proliferation, enhancing apoptosis and suppressing GBM growth.
Fig 13. Azt/nimbolide suppresses tumor growth in vivo
(A) Representative digital photograph showing U87EGFRvIII tumor growth in mice injected with EtOH (red arrow) or Azt (blue arrow). (B) Digital photographs of tumors injected with EtOH or Azt were dissected out at the end of the experiment. (C) Growth of tumor xenografts in Nu/Nu mice (n=8) initiated by U87EGFRvIII cells and treated with EtOH or Azt. Treatment started following tumor growth for seven days. Injection schedule and tumor volume measurement at indicated days are shown. (DG) H&E of tumors injected with EtOH or Azt. Note: Cells in different stages of cell division (yellow arrowheads) in (D) which is significantly reduced in (E), and marked increase in cells with condensed/fragmented chromatin and pycnotic nuclei (indicative of dead cells) in (G) compared to (F). Immunohistochemistry using Ki67 antibody showing proliferating cells (H, I), quantitation of Ki67+ cells (J), using cleaved Caspase 3 (CC3) antibody showing apoptotic cells (K, L) and quantitation of CC3+ cells (M) in EtOH and Azt treated tumors. Immunohistochemistry showing phosphorylated levels of Akt (N, O), Erk1/2 (P, Q) and STAT3 (R, S) in EtOH and Azt treated tumors. *p<0.0001.
5. DISCUSSION

GBM remains one of the most lethal forms of human cancers as less than 5% of patients diagnosed with GBM will survive more than 5 years (73). Despite comprehensive sequencing analysis and improved understanding of the core genetic pathways altered in GBM (74), there are still many unresolved questions relevant to the etiology of this disease. Single agent therapy targeting one selective pathway has not yet resulted in the desired therapeutic outcomes. Accordingly, standard of care therapy only offers progression free survival by about 20% (75). Therefore, new therapeutic strategies must be explored to effectively enhance therapeutic options for GBM patients. The primary objective of this study was to examine cytotoxic efficacy of Azt and its principal cytotoxic component nimbolide on GBM survival and growth. The results obtained demonstrate that Azt/nimbolide induces robust cytotoxic efficacy in GBM models in vitro and in vivo. In comparison to other anticancer agents including TMZ, PI3K and mTOR inhibitors, Azt/nimbolide appears most potent of all the agents tested so far.

While all GBM cells were susceptible to Azt/nimbolide-induced cytotoxicity, GBM cells expressing the EGFRvIII oncogene showed greater sensitivity to this agent. The IC50 of nimbolide on this cell was found to be 3.0 µM as compared to the values in other cell lines. Azt/nimbolide arrested GBM cells mostly at the G1-S border. The magnitude of cell cycle arrest was dependent on cell line and
was most pronounced in U87EGFRvIII cells. The extent of RB hypophosphorylation by Azt/nimbolide in this cell line was far greater than that in other GBM cells (Fig 11B; 12B), which might partly explain the more dramatic G1-S arrest of these cells. Hyperactivation of signaling pathways by oncogenes causes oncogenic stress (76), and cancer cells have evolved mechanisms to counter such stress to remain viable. It is possible that Azt/nimbolide perturbs one or more of these anti-stress pathways in the U87EGFRvIII cells.

Anchorage-independent growth is a classical assay to interrogate invasiveness and metastatic potential of cancer cells (77). U87 and U87EGFRvIII cells are highly invasive in vivo (data from many other labs and our own data not shown) and forms colonies in vitro. The ability of Azt/nimbolide to prevent colony formation was remarkable and dose-dependent. At the highest dose (20µM nimbolide or 4µg/µl Azt), large colonies were virtually undetectable. This suggests that this agent, upon sufficient systemic activity retention, might inhibit tissue invasion and metastasis.

The mechanisms of cell death induced by Azt/nimbolide remain unclear. Depending on cell type, Azt/nimbolide caused high to modest apoptosis of cancer cells including HeLa (cervical carcinoma), THP1, U937, HL60, B16 (leukemia) (78, 69) and HT29 (colon cancer) cells (1). In one study Azt/nimbolide caused apoptosis in leukemia, lymphoma, human embryonic
kidney, multiple myeloma, breast cancer and human squamous cell carcinoma by suppressing cytokine-induced NF-κB activation (66). In GBM cells, Azt/nimbolide did not cause extensive apoptosis in vitro. However, in vivo, we did observe many pycnotic nuclei and positive staining for cleaved Caspase 3. This implies that the tumor microenvironment, including tumor hypoxia, potentiates Azt-dependent apoptosis. Downstream of PI3K, Akt plays a major role in promoting cell survival by stabilizing the Bcl2 protein (79) and increasing its transcription (80). It is possible that similar to other studies (65) downregulation of Bcl2 by Azt/nimbolide in our study is due to its inhibitory action of Akt phosphorylation.

Signaling pathways that are frequently dysregulated in GBM include the EGFR/EGFRvIII driven PI3K-Akt pathway, RAS-MAP kinase pathway, JAK-STAT pathway and the RB pathway (29, 74). It is therefore remarkable that Azt/nimbolide suppressed all these pathways with the greatest effect observed in GBM cells expressing the EGFRvIII oncogene. Azt/nimbolide, however, did show some specificity since it suppressed the above pathways without inhibiting mTOR and without changing the total protein levels of several cell cycle regulators including Cyclin D, Cyclin B, P27 and P21. The three adult GBM cells that we primarily used in this study are all homozygous null for the G1-S cell cycle inhibitor INK4a/ARF (CDKN2A in humans) that inhibits CDK4/6, the primary kinase that phosphorylates and inactivates RB. Absence of
INK4a/ARF therefore leads to unchecked CDK4/6 activity in these GBM cells. Thus, it is of high significance that Azt/nimbolide directly inhibits CDK4/6 activity leading to RB hypophosphorylation and G1-S arrest, particularly in GBM cells expressing the oncogene EGFRvIII.

Studies showing the in vivo cytotoxicity of Azt/nimbolide are limited. The LD50 of Azt was found to be 4.57 mg/g body weight in acute toxicity studies in rodents (81). In this study, we found that acute exposure of GBM cells in vitro to Azt prevents tumor formation in vivo. We attempted to convert Azt into a water-soluble delivery form by evaporating EtOH and resuspending the remaining solid materials in the solvent. Unfortunately, the resulting material was ineffective in killing GBM cells in vitro. Therefore, we had to rely on the EtOH soluble fraction for in vivo studies. This limited our efforts to conduct IP injections due to systemic toxicity of EtOH. However, we demonstrated that intratumor injection of a very low dose of Azt (0.34 µg/g body weight) significantly suppressed tumor growth. Grade IV gliomas or GBMs are histologically characterized by hypercellularity and presence of extensive mitotic activity. Azt reduced hyper cellularity, inhibited proliferation and induced apoptosis in GBM tumor xenografts. It also inhibited growth factor signaling in vivo as revealed by the reduced staining of phosphorylated Akt, Erk1/2 and STAT3 in Azt injected tumors.
6. CONCLUSION

The results from this study demonstrate that Azt/nimbolide is a potent cytotoxic agent that exerts antiproliferative and apoptotic effect in GBM in vitro and in vivo. It suppresses GBM viability and suppresses tumor growth by inhibiting CDK4/6 activity leading to RB hypophosphorylation and cell cycle arrest and by inhibiting growth factor pathways hyperactivated in GBM including the PI3K-Akt, MAP kinase and JAK-STAT pathways. Further studies are needed to explore solubility of Azt in aqueous solutions, examine the bioavailability of Azt and nimbolide and determine their therapeutic efficacy as a single agent or in combination with chemotherapy and radiation in orthotopic mouse models of human GBM and other cancers.

FUTURE DIRECTIONS

The primary goal of the present study was to determine in vivo and in-vitro efficacy of the Azt on glioma cell viability and proliferation as well as to elucidate its mechanism of action. To explore future clinical applications, however, active compounds of Azt must be formulated as a pharmacologically acceptable dosage form that allows safe patient administration without significant loss of pharmacodynamic efficacy.

Nimbolide, the main active ingredient hypothesized to facilitate cytotoxic effects reported in this study, is a lipophilic limonoid that requires the presence of organic solvents for efficient extraction. In our experiments, dried leaf
powder was macerated for 7 days with EtOH to extract pharmacologically active ingredients into solution. However, in the pre-clinical experiments, mice injected intra peritoneally with 62.5 µl EtOH died immediately after injections. The intraperitoneal and intravenous LD\textsubscript{50} of ethanol in mice is 933 mg/kg and 1973 mg/kg respectively. This indicates that not more than 29.5 µl IP or 62.5 µl IV administration of EtOH is possible in our animal models. To assure safety and to confirm that the toxic effects are due to the extract alone, it is recommended to administer only about a quarter of the above stated volumes depending on the route of administration. Thus, appropriately controlled monitoring of the rate or route of administration and the dose can be tried to verify if this toxicity can be avoided.

Alternatively, an essential aspect of future development of this project is to establish a drug delivery system without ethanol that effectively limits proliferation of GBM cells \textit{in vivo} after standard intravenous administration. EtOH used to extract therapeutically active components for neem leaves can be removed under vacuum. The subsequent aim, however is to solubilize the lipophilic components such as nimbolide in an aqueous based vehicle. Designing dosage forms of Neem that exhibit high bioavailability and allow low dose administration as compared to standard therapeutic drugs like doxorubicin has been recognized as a major challenge due to lipophilicity of its active components. Surfactant based drug delivery systems may be used to overcome
this limitation. Tween-80 and Labrafac are pharmacologically acceptable surfactants that are clinically used to solubilize lipophilic drugs and can be tested for effective solubilization of nimbolide or other neem components. In addition to surfactants, co-solvents such as polyethylene glycols and hydrotropes such as Transcutol P represent powerful excipients to facilitate effective solubilization of lipophilic components in a hydrophilic solvent. In combination with oil phases, these excipients allow fabrication of self-microemulsifying drug delivery systems (SMEDDS) which represent liquid dosage forms consisting of a mixture of drug, oil, surfactant and co-surfactant. Gentle mixing of these ingredients in an aqueous media facilitates formulation of an oil-in-water microemulsion without the use of high physical forces. Droplets with a mean size of <100 nm indicate successful preparation. Many literature examples demonstrate advantages of SMEDDS with respect to drug solubility and improved absorption and bioavailability of hydrophobic drugs (93, 94, and 95). Sub-microemulsions are novel drug delivery systems which have been employed as carriers for lipid soluble drugs, especially anti-cancer drugs, anti-inflammatory drugs and anesthetic drugs (96, 97 and 99). These preparations have a number of advantages like increased stability, improved in vivo drug distribution, and improved target function and intravenous administration of hydrophobic drugs (98, 99).
Consequently, future work should focus on developing surfactant-based SMEDDS containing the anticancer extract of neem leaf for the possible treatment of solid tumors such as glioblastomas. We hypothesize that this drug delivery system, after systematic \textit{in vitro} studies, can then be tested in suitable animal models and, subsequently, in patients. Various surfactant and co-solvent combinations should be tested at different concentrations for their individual cytotoxicity on the cells in order to select the safest components for the design of a SMEDDS. Soybean oil, which is used commonly in parenteral preparations, may be a suitable starting point as the oil phase. In addition to surfactants and the oil phase, a hydrotrope such as Transcutol P could greatly enhance the solubilization power of the surface active mixtures, which ultimately results in increased stability of SMEDDS. Systematic \textit{in vitro} studies on cytotoxic efficacy of these systems on cancer cell lines are anticipated to pave the way for more extensive \textit{in vivo} studies with substantial relevance for future clinical applications in GBM patients.

Consequently, future work should focus at developing surfactant-based SMEDDS containing the anticancer extract of neem leaf for the possible treatment of solid tumors such as glioblastomas. It can be hypothesized that this drug delivery system, after systematic \textit{in vitro} studies, can then be tested in suitable animal models and, subsequently, in patients. Various surfactant and co-solvent combinations should be tested at different concentrations for their
individual cytotoxicity on the cells in order to select the safest components for
the design of a SMEDDS. Soybean oil, which is used commonly in parenteral
preparations, may be a suitable starting point as the oil phase. In addition to
surfactants and the oil phase, a hydrotrope such as Transcutol P could greatly
enhance the solubilization power of the surface active mixtures which,
ultimately, results in increased stability of SMEDDS. Systematic in vitro studies
on cytotoxic efficacy of these systems on cancer cell lines is anticipated to pave
the way for more extensive in vivo studies with substantial relevance for future
clinical applications in GBM patients.
7. BIBLIOGRAPHY


analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1."

*Cancer Cell* 17(1): 98-110.


[31] "Entrez Gene: PTEN phosphatase and tensin homolog (mutated in multiple advanced cancers 1)"


437.


52(5): 922-926.


