IN VIVO AND IN VITRO EFFECTS OF AZIDOTHYMIDINE ON NEURAL STEM/PROGENITOR CELLS IN MOUSE

By

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To my Mom Hacer Cakiroglu, for her endless love and support that I will forever be thankful for.
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<td>ACTG 076</td>
<td>AIDS Clinical Trials Group Protocol 076</td>
</tr>
<tr>
<td>ADC</td>
<td>AIDS Dementia Complex</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AZT</td>
<td>Azidothymidine - 3’-azido-3’-deoxythymidine</td>
</tr>
<tr>
<td>AZT-DP</td>
<td>AZT-diphosphate</td>
</tr>
<tr>
<td>AZT-MP</td>
<td>AZT-monophosphate</td>
</tr>
<tr>
<td>AZT-TP</td>
<td>AZT-triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood-Cerebrospinal Fluid Barrier</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine - 5-bromo-3’-deoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPE</td>
<td>CNS Penetration Effectiveness</td>
</tr>
<tr>
<td>CS</td>
<td>Cesarean Section</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>dT-TP</td>
<td>Deoxythymidine Triphosphate</td>
</tr>
<tr>
<td>E12</td>
<td>Embryonic Day 12</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV Associated Dementia</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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<td>-----------</td>
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<tr>
<td>HTLV-III</td>
<td>Human T-Lymphotrophic Virus Type III</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Inhibition concentrations</td>
</tr>
<tr>
<td>LAV</td>
<td>Lymphadenopathy-Associated Virus</td>
</tr>
<tr>
<td>MASC</td>
<td>Multipotent Astrocytic Stem Cell</td>
</tr>
<tr>
<td>MND</td>
<td>Mild Neurocognitive Disorder</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NCFC</td>
<td>Neural Colony Forming Cell</td>
</tr>
<tr>
<td>NCFCA</td>
<td>Neural Colony Forming Cell Assay</td>
</tr>
<tr>
<td>NDK</td>
<td>Nucleoside Diphosphate Kinase</td>
</tr>
<tr>
<td>NICHD</td>
<td>National Institute of Child Health and Human Development</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural Progenitor Cell</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NS</td>
<td>Neurosphere</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural Stem Cell</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory Bulb</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>P2</td>
<td>Postnatal Day 2</td>
</tr>
<tr>
<td>Pol-γ</td>
<td>Polymerase Gamma</td>
</tr>
<tr>
<td>rhEGF</td>
<td>Human Epidermal Growth Factor</td>
</tr>
<tr>
<td>RLV</td>
<td>Rauscher Murine Leukemia Virus Complex</td>
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<tr>
<td>SAβGal</td>
<td>Senescence-Associated β-Galactosidase</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>TK1</td>
<td>Thymidine Kinase 1</td>
</tr>
<tr>
<td>TK2</td>
<td>Thymidine Kinase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TMPK</td>
<td>Thymidylate Kinase</td>
</tr>
<tr>
<td>T-TP</td>
<td>Thymidine Triphosphate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5 Bromo-4-chloro-3-indolyl B-Dgalactoside</td>
</tr>
<tr>
<td>ZDV</td>
<td>Zidovudine</td>
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IN VIVO AND IN VITRO EFFECTS OF AZIDOTHYMIDINE ON NEURAL STEM/PROGENITOR CELLS IN MOUSE

By

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Azidothymidine (3’-azido-3’-deoxythymidine; AZT) is a nucleoside reverse transcriptase inhibitor that has been used in the treatment and prevention of human immunodeficiency virus-1 (HIV-1). Even though there is no active transportation of AZT across the blood brain barrier, AZT is shown to accumulate in high levels in cerebrospinal fluid (CSF) with limited penetration into the parenchyma. Due to the close anatomical proximity of the neurogenic niches to the ventricular system, we suggest that passive diffusion from CSF may be sufficient to expose the neurogenic cells to biologically relevant levels of AZT that may be sufficient to perturb the normal production and/or survival of newly-generated neurons. In turn, this perturbed neurogenesis contribute to, or be the basis of many of the neurological deficits seen in some cases of AIDS.

In order to assess the effects of clinically relevant AZT regimens on neuronal production, we have employed in vitro and in vivo models of mouse neurogenesis. Our in vitro results show that AZT reduces the expansion potential of neural stem/progenitor cells, eventually inducing a senescent phenotype. In addition, AZT treated cells display impaired differentiation potential and increased susceptibility to apoptosis. In vivo, our
AZT administration paradigm mimicking dosing regimens that administered to human patients revealed a remarkable decrease in the survival of newly formed cells in subventricular zone (SVZ) in contrast to the short term neurogenesis which is not affected significantly within the dentate gyrus and SVZ of adult mice. Finally, our analysis of in utero exposure demonstrates that AZT affects SVZ stem/progenitor cells so their neurogenic potential is perturbed and the expansion potential of cultured neural stem/progenitor cells from treated offspring is decreased.

Together, these data reveal uncharacterized negative consequences of AZT treatment on neural stem/progenitor cells. Given that HIV infection leads to the development of neurological deficits, and that human HIV (+) patients are usually treated with AZT over many years, it is important to determine to what extent AZT regimens might perturb normal levels of neurogenesis to exacerbate or contribute to these neurological problems. We suggest that in case of increased delivery of AZT into the brain, a direct exposure to the CNS would cause more dramatic changes as we have shown with in vitro cell culture systems. We expect our results will reveal new insights regarding the effect of AZT on stem/progenitor cell functioning, and the development of new treatment approaches to prevent the HIV infection in CNS.
CHAPTER 1
INTRODUCTION

Neurogenesis in the Adult Mammalian Brain

History

Cajal’s statement “Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be generated, It is for the science of the future to change, if possible, this harsh decree.” in 1913, has been so influential that it took almost a century to break this long-held dogma.

Because most cells in the mammalian central nervous system (CNS) are in a quiescent state, neurogenesis was for many decades believed to occur only during embryonic and early postnatal development. In 1962, by using intracranial injection of thymidine-H3, Altman showed the presence of proliferating glia, neurons and neuroblasts in damaged brain areas of adult rats for the first time (Altman, 1962). More than three decades later, the discovery of neurogenesis in the dentate gyrus of adult humans had an important impact on the field (Eriksson et al., 1998). It is now known that in the adult mammalian brain there are two main neurogenic niches, the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus. Throughout life the neural stem cells in the adult mammalian brain are able to generate functional cells regulated by signals under physiological and pathological conditions (Alvarez-Buylla and Lim, 2004; Zhao et al., 2008).
Neurogenic Niches in the Adult Mammalian Brain

Neural stem cells (NSCs) are self-renewing, multipotent cells that are located in the persistent neurogenic niches of the SVZ of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus. In the SVZ, there are three precursor cell populations: Neural stem cells compromising slowly dividing, GFAP+ SVZ astrocytes (type B); rapidly dividing Dlx2+ transit amplifying cells (type C); and Dlx2+/β-III-tubulin+ migratory neuroblasts (type A). Type A neuroblasts migrate along the rostral migratory stream to reach the olfactory bulb (OB) where they then migrate radially and differentiate into granule and periglomerular cells (Laywell et al., 2000). In the developing brain, the embryonic NSCs, which are radial glia, reside in the ventricular zone directly contacting both the pial and ventricular surfaces. On the other hand, the neural stem cells (type B) in the SVZ of adult brain derived from radial glia are separated from the anterolateral walls of the adult lateral ventricle by a single layer of ependymal cells. Recently, it has been shown that the SVZ neural stem cells (type B) have an apical ending which directly contacts with the ventricle and a basal process ending on blood vessels (Mirzadeh et al., 2008). Moreover, the NSCs contacting blood vessels lack astrocyte endfeet, and pericyte coverage (Tavazoie et al., 2008). This modified blood brain barrier structure exposes SVZ stem cells to various signals from the vascular system.

The SGZ niche has two precursor cell populations: Infrequently dividing, GFAP+/Sox2+ radial NSCs (type 1); and mitotically active and Sox2+ nonradial NSCs (type 2). A subpopulation of type 2 cells have shown to self-renew and to give rise to a neuron and an astrocyte revealing their stem cell properties (Suh et al., 2007). The
neuroblasts derived from type 2 cells migrate into the granule cell layer (GCL) and mature into neurons.

Especially, the SVZ has been shown to have the largest population of proliferating cells in the adult brain of rodents, monkeys and humans (Altman, 1963; Lois & Alvarez-Buylla, 1994; Gould et al., 2001; van Praag et al., 2002; Ramirez-Amaya et al., 2006; Zhao et al., 2006). In the mouse SVZ the estimated number of cells generated bilaterally is about 30,000/day (Alvarez-Buylla et al., 2002; Abrous et al., 2005).

**Neural Stem Cell Functioning**

It is now clear that neurogenic regions in the adult mammalian brain continue to produce new neurons throughout life. The newly generated neurons arise from the neural progenitor cells that reside in the subventricular zone and in the subgranular zone of the dentate gyrus.

Neurogenesis in dentate gyrus has been shown to be modulated by physical activity, seizures and aging (Lugert et al.; Fabel & Kempermann, 2008). There is evidence that hippocampal neurogenesis is sensitive to environmental insults leading to impairments in synaptic plasticity, learning and cognition (Kempermann et al., 1997; Kempermann & Gage, 1999; van Praag et al., 1999; Kronenberg et al., 2003; Steiner et al., 2008; Zhao & Overstreet-Wadiche, 2008; Petrus et al., 2009). In addition to hippocampal neurogenesis, it is shown that an odor-enriched environment enhances neurogenesis from SVZ, and improves olfactory memory without upregulating hippocampal neurogenesis (Rochefort et al., 2002; Alonso et al., 2006; Lledo et al., 2006; Alonso et al., 2008). Moreover, in neural cell adhesion molecule (NCAM) deficient mice, where the migration of OB neuron precursors is reduced, the discrimination between odors is shown to be impaired (Gheusi et al., 2000; Gheusi & Rochefort,
2002). Furthermore, stroke induced neurogenesis in SVZ and SGZ has been shown both in experimental animals and human patients (Ekonomou et al.; Liu et al., 1998; Kee et al., 2001; Jin et al., 2004; Wang et al., 2005; Jin et al., 2006; Macas et al., 2006; Minger et al., 2007).

**In vitro models of Neurogenesis**

Understanding the biological features of neural stem cells and their progeny is one of the main goals in the field of neuroscience. The dogma that there are not new neurons produced in the adult mammalian brain has persisted for more than a century mainly because of the lack of methods to demonstrate neural stem cells which are capable of generating new cells.

In 1959, Sidman et al. had introduced the $[^{3}\text{H}]$-thymidine autoradiography method to label the DNA of dividing cells (Sidman et al., 1959). Using this technique, Smart showed the production of new neurons in the postnatal mouse brain for the first time. On the other hand, he was not able to prove the generation of new neurons in the adult brain. In 1962, Altman had published his first article showing new neuron formation in the adult rat brain (Altman, 1962). However his series of publications reporting $[^{3}\text{H}]$-thymidine labeled cells in dentate gyrus (Altman & Das, 1965), neocortex (Altman, 1963; Altman & Das, 1966) and olfactory bulb (Altman, 1969) of adult rats had been largely unappreciated (Ming & Song, 2005). In 1977, Kaplan and Hinds showed ultrastructural evidence for new neurons in dentate gyrus and olfactory bulb of adult rats by using electron microscopy in addition to $[^{3}\text{H}]$-thymidine technique for the first time (Kaplan & Hinds, 1977). In 1982, the introduction of 5-bromo-3′-deoxyuridine (bromodeoxyuridine, BrdU) labeling became a very important development to prove neurogenesis in the adult brain. BrdU is synthetic thymidine analogue which
incorporates into DNA during S-phase of mitosis and labels proliferating cells and their progeny. Since BrdU labeling can be detected by immunocytochemical methods, it makes identification of newly generated cells with their cell-specific markers possible.

Finally, in 1992, Reynolds and Weiss had first isolated neural stem and progenitor cells from adult rodent CNS and expanded them in culture conditions (Reynolds & Weiss, 1992). In addition, in 1999, Kukekov and colleagues showed that adult human brain also has neurogenic cells that can produce neuronal and glial progeny under certain in vitro growth conditions (Kukekov et al., 1999). The isolation and in vitro analysis of neurogenic cells from the adult brain allows characterization of mechanisms of the function and regulation of neurogenic stem/progenitor cells.

A stem cell is currently defined as an undifferentiated cell that exhibits the ability to proliferate, to self renew, and to differentiate into multiple and distinct lineages. On the other hand, progenitor cells are mitotic cells with faster dividing cell cycle that maintain the ability to proliferate and to give rise to terminally differentiated cells but are not capable of indefinite self-renewal.

Currently, the neural stem and progenitor systems isolated from SVZ can be cultured and maintained in vitro as monolayers of multipotent astrocytic stem cells (MASC) and aggregates of clonal stem/progenitor cells as known as neurospheres (NS). In addition, recently developed neural colony forming cell assay (NCFCA) allows us to distinguish neural stem and progenitor cells on the basis of their proliferative potential (Louis et al., 2008). These in vitro model systems for the isolation, expansion and differentiation of NSCs provide understanding of the biology of adult stem cells.
MASCs can be isolated from dissociated SVZ tissue and form a monolayer after culture on adhesive substrate such as laminin in the presence of EGF, FGF and serum. These highly proliferative neurogenic astrocyte cultures consisting of astrocytes, neurons and microglia can be expanded for >75 population doublings (Scheffler et al., 2005; Marshall et al., 2008). Furthermore, the withdrawal of mitogens, EGF and FGF, and serum induces differentiation resulting in a rapid generation of B-III-tubulin expressing neuroblasts. In addition, when cultured on nonadhesive surface in the presence of EGF and FGF, MASCs can generate multipotent neurospheres. Neurosphere culture is another in vitro cell culture system we use. Neural stem cells isolated from SVZ can be maintained in a serum free medium supplemented with mitogens EGF, FGF and heparin. The spherical aggregate of clonal cells, called neurospheres, display the stem cell features of multipotency, serial expansion and self renewal. The neurosphere assay is the most frequently adopted technique to isolate and investigate the biology of neural stem cells (Reynolds & Rietze, 2005). This method is shown to overestimate stem cell number since not all of the neurospheres are derived from stem cells. Indeed, it is shown that neurospheres are heterogenous including stem cells, proliferating neural progenitor cells, postmitotic neurons and glia (Suslov et al., 2002). However another cell culture system, neural colony forming cell assay (NCFCA), allows us to distinguish stem and progenitor cells on the basis of their proliferative potential. A single cell suspension isolated from SVZ or neurospheres can be cultured on nonadhesive surface, in serum-free, semi-solid collagen media supplemented with mitogens, EGF, FGF and heparin. Clonally derived colonies are formed based on the stem and progenitor cells’ proliferative potentials. Stem cells with high proliferative
potential form the largest sized colonies (>2mm in diameter) while progenitor cells with low proliferative potential form smaller colonies (<2mm in diameter). Altogether, these in vitro cell culture systems allow us to model postnatal/adult neurogenesis and to monitor the molecular and cellular features of stem cells.

Azidothymidine

Background

Azidothymidine (3'-azido-3'-deoxythymidine (AZT); zidovudine (ZDV); Retrovir™, formerly BW A509U) is a synthetic analogue of thymidine in which the 3-hydroxyl group is replaced by an azido group (Figure 1-1). AZT was first synthesized in 1964 against oncoviruses however it was shown to be ineffective as an antineoplastic drug (Dube & Ostertag, 1991). Ten years later, it was shown that AZT inhibits virus replication in a mouse retrovirus culture system (Ostertag et al., 1974). Later in 1985, Mitsuya et al. showed that triphosphorylated AZT inhibits the reverse transcriptase of HTLV-III/LAV (human T-lymphotrophic virus type III/lymphadenopathy-associated virus) – later HIV- in H9 cells, and suggested the development of AZT as a potential treatment for HIV infection in humans (Mitsuya et al., 1985). In 1986, Ruprecht et al. showed that AZT treatment of mice with Rauscher murine leukemia virosus complex (RLV) suppresses viraemia and prolongs lifespan (Ruprecht et al., 1986). Furman et al. showed that AZT-triphosphate inhibits the purified HIV Reverse Transcriptase about 100 times more effectively than it inhibits cellular DNA polymerase α of immortalized human lymphocytes (H9 cells) in 1986. In addition, half inhibitory dose (IC₅₀) for cellular replication is 10,000 times higher than the IC₅₀ for HIV replication (Furman et al., 1986). After the first clinical trial performed in 1986 (Yarchoan et al., 1986), Fishl et al. suggested that AZT could be safely administered to HIV (+) patients, and it could
prolong the life of patients with AIDS (Fischl et al., 1987). Finally, in 1987 the Food and Drug Administration approved AZT for use against HIV/AIDS.

Since then, AZT has been used in the treatment and prevention of human immunodeficiency virus-1 (HIV-1) infection alone or in combination with other antiviral agents as an integral part of the Highly Active Antiretroviral Therapy (HAART) protocols, and human T-cell lymphotropic virus type I (HTLV)-I-associated adult T-cell leukemia/lymphoma (Falchetti et al., 2005). In addition, AZT monotherapy has been recommended for use in pregnancy to reduce vertical transmission of HIV-1 from mother to fetus (Olivero, 2007) even though it is classified in Food and Drug Administration (FDA) Pregnancy Category C which states that safety of AZT usage in human pregnancy has not been determined, and it should not be used unless the potential benefit outweighs the potential risk to the fetus.

**Mechanism of Action**

As a thymidine analog, AZT interacts with the same metabolic enzymes as thymidine. The therapeutic activity of AZT depends on the level of its conversion to the active form, AZT-triphosphate (AZT-TP), by a three step cascade of phosphorylation which can be catalyzed in either cytoplasm or the mitochondrion. AZT is first phosphorylated to AZT-monophosphate (AZT-MP) in a reaction catalyzed by either cytosolic thymidine kinase 1 (TK1) or mitochondrial thymidine kinase 2 (TK2). TK1 is more abundant in cells with rapid mitotic turnover and its concentration is increased during the S-phase (Lewis et al., 2003). The phosphorylation of AZT-MP to AZT-diphosphate (AZT-DP) is catalyzed by thymidylate kinase (TMPK). Finally, the phosphorylation of AZT-DP to the active form AZT-TP is catalyzed by nucleoside diphosphate kinase (NDK). TMPK plays a rate-determining role in the conversion of
AZT-MP to AZT-TP. Since the P-loop in the enzyme is prevented by the 3’-azido group of AZT-MP from adopting the necessary conformational change, AZT-MP accumulates in cells exposed to AZT (Lavie et al., 1997; Ostermann et al., 2000). This leads a decrease in the catalytic efficacy of the enzyme and depletion of thymidine triphosphate (T-TP) levels, giving AZT-TP a competitive advantage over T-TP for the incorporation into the growing HIV DNA by HIV reverse transcriptase (Bradshaw et al., 2005). After incorporation into the proviral DNA, the AZT-TP terminates the formation of DNA chain, and reverse transcription starts from the beginning following the release of incomplete DNA.

**Therapeutic Usage in Pregnancy**

At the end of 2008, 33.4 million people worldwide were estimated to be living with HIV infection. About 2.7 million of adults and children are newly infected and 2 million adults and children died due to AIDS. At the end of 2009 there are an estimated 2.1 million children living with HIV, most of who were infected by their mothers. The current case number of HIV-1/AIDS infected pregnant women a year in United State is about 7000 (Olivero, 2008). It is estimated that approximately 25% to 48% of infants born to HIV infected mothers may become HIV-1 infected through breastfeeding (Mbori-Ngacha et al., 2001; Thior et al., 2006). In 1994, the AIDS Clinical Trials Group Protocol 076 (ACTG 076) study established that AZT monotherapy plays a beneficial role in reducing mother to child transmission of HIV (Connor et al., 1994). In a nonbreastfeeding population, AZT monotherapy was administered at 14-34 weeks of gestation (600mg/day), continuous intravenous infusion during labor (2mg/kg loading dose followed by 1/mg/kg/h) and 6 weeks of oral dosing to the newborn reduced the mother to child viral transmission rate from 25.5% to 8.3%. Since then, AZT has been used in
the prevention of mother to child transmission of HIV-1 and it remains the only licensed antiretroviral for use during pregnancy even AZT is categorized in FDA Pregnancy C (Walker et al., 2007; Durand-Gasselin et al., 2008; Read et al., 2008; Foster et al., 2009). In the absence of prenatal treatment, 5–10% of infants born to HIV-infected women are infected across the placenta, 10–20% of infants are infected from exposure at the time of delivery and 5–20% of infants are infected through breast feeding which is responsible of 50% of HIV infections in children in Africa (Wade et al., 1998; De Cock et al., 2000).

AZT is metabolized by the liver through glucuronidation resulting in bioavailability about 63%. Its plasma peak concentration is achieved in 1h and its half-life is about 1.1 h in non-pregnant adults. It is shown that AZT crosses the placenta rapidly with similar concentrations in maternal plasma, amniotic fluid and cord blood plasma. The AZT monotherapy regimen is shown to result in an average plasma concentration of 0.82 μg/ml in the mother and 0.75 μg/ml in the newborn (Capparelli et al., 2005).

Busidan et al. have shown that after a single dose of 150 mg/kg AZT administration to E20 pregnant rats and P20 pups, the distribution of AZT in fetus brain is heterogeneous with relatively greater amounts of AZT in the periventricular area. On the other hand, there was less exposure of the brain in the P20 pups than at E20 fetuses. They hypothesized that in the E20 fetuses, the efflux transporter of AZT has either not developed or is not yet efficient at removing AZT from the brain (Busidan et al., 2001).

In addition to the perinatal genotoxicity findings, there have been neurobehavioral studies focusing on the possible toxic effects of AZT administration during development.
Prenatal AZT exposure has been shown to interfere with CNS development and have a long term neurobehavioral consequences such as impaired locomotor activity, deficit in learning and spatial tasks, deficits in social, agonistic and investigative behavior, and long-term functional alterations within sensorimotor reflexes (Calamandrei et al., 1999b; Rondinini et al., 1999; Venerosi et al., 2000; Ricceri et al., 2001; Calamandrei et al., 2002b; Venerosi et al., 2003; Levin et al., 2004; Melnick et al., 2005; Venerosi et al., 2005).

Since AZT has been shown to distribute to the CNS of a developing brain, it is important to examine neurogenic potential of SVZ and dentate gyrus stem/progenitor cells which may be particularly vulnerable to the toxic effects of AZT due to these regions anatomical proximity to the ventricular surfaces.

**Dosage and Administration**

In the present day, for human adults the total recommended daily amount of AZT is 600 mg, which is about 10mg/kg, resulting in a steady-state serum AZT concentration of 0.8µM (Fletcher et al., 2002). While AZT's plasma half life is 0.5-3 hours, its active metabolite's (AZT-TP) intracellular half-life is 7 hours. The levels of AZT in brain tissue have been shown to be IC₅₀ (0.003–0.013µg/ml) (Cook et al., 2005).

Moreover, AZT monotherapy at 600mg/day dose is administered at 14-34 weeks of gestation, continuous intravenous infusion during labor (2mg/kg loading dose followed by 1/mg/kg/h) and 6 weeks of oral dosing to the newborn results in an average plasma concentration of 0.82 µg/ml in the mother and 0.75 µg/ml in the newborn (Capparelli et al., 2005). For human neonate, the recommended dose of AZT is 2 mg/kg every 6 hr (8 mg/kg/day) (Witt et al., 2004). FDA’s recommended pediatric dosage of AZT is between 24 mg/kg/day and 600mg/kg/day as shown in Table 1. Alternatively,
dosing for AZT can be based on body surface area for pediatric patients. The recommended oral dose is 480mg/m\(^2\)/day.

In order to equate mouse and human doses, we used mg/m\(^2\) conversion factors based on FDA’s recommendation. Accordingly, 10mg/kg dose, which is 600mg AZT/day, administered to human with 1.1710m\(^2\) body surface area would be equal to 133mg/kg dose administered to mouse with 0.007m\(^2\) body surface area. In addition, AZT concentrations at 500 to 1500 mg/day correspond to 20 to 60 µM of AZT (Brown et al., 2003).

**Toxicity Mechanisms**

Although AZT’s effect is proven in controlling viral infection in adult patients and in reducing vertical viral transmission, several studies showed adverse effects of AZT such as bone marrow suppression, pancytopenia, anemia, macrocytosis, cardiomyopathy, hepatic steatosis, fatal lactic acidosis, myopathy, peripheral neuropathy, distal symmetrical neuropathy, and carcinogenicity (Ayers et al., 1996; Chow et al., 1997; Zhang et al., 1998; Diwan et al., 1999; Anderson et al., 2003; Lee et al., 2003; Lewis et al., 2003; Lai et al., 2004; Lewis et al., 2004; Torres et al., 2007).

It is difficult to distinguish the toxicity of AZT from that due to HIV infection in patients receiving HAART. Ippolito et al. examined the short term toxicity of AZT given as prophylaxis to HIV-exposed healthcare workers. They showed that 49% of 674 healthcare workers given 300 to 3000mg/day AZT had at least one adverse effect, and 20% of these discontinued prophylaxis because of side effects. In addition, they found that all side effects were frequent, mild, dose related and reversible after the prophylaxis was stopped (Ippolito & Puro, 1997). Finally, it was shown that pathologies such as myopathies, cardiomyopathy, hepatotoxicity usually resolve when AZT is removed from
the patient’s therapy regimen, suggesting these pathologies are due to AZT and not symptoms of AIDS (Lynx & McKee, 2006).

The exact mechanism by which AZT causes toxicity is not known. The inhibition of mitochondrial DNA (mtDNA) polymerase gamma (pol-\(\gamma\)) has been suggested as the mechanism for AZT-related adverse effects due to the fact that the strongest interaction of AZT with the host polymerases is with mtDNA pol-\(\gamma\) followed by DNA polymerase \(\beta\), DNA polymerase \(\alpha\) and DNA polymerase \(\varepsilon\). In addition to the DNA pol-\(\gamma\) hypothesis, suggesting that the inhibition of mtDNA pol-\(\gamma\) causes the depletion of mtDNA and mitochondrial dysfunction, the mitochondrial dysfunction hypothesis includes the pathophysiological results of mtDNA mutations and mitochondrial stress.

AZT has been shown to decrease mtDNA levels both in vivo in experimental animals and clinically in humans (Lewis et al., 1992). AZT-MP accumulates at high concentrations intracellularly because of the fact that AZT-MP prevents the conformational change in the TMPK which also catalyzes TMP. As a result, compared to the phosphorylation rate of TMP, the phosphorylation rate of AZT-MP to AZT-DP by TMPK is about 60-fold low (Papadopulos-Eleopulos et al., 1995; Brundiers et al., 1999; Ostermann et al., 2000). While 94% of AZT metabolites are AZT-MP, the concentration of active AZT-TP and AZT-DP consists only 6% of the metabolites (Lavie & Konrad, 2004; von Kleist & Huisinga, 2009). The depletion of TTP levels caused by AZT-MP and competitive inhibition of TTP by AZT-TP are generally accepted factors leading to toxicity (Samuels, 2006; von Kleist & Huisinga, 2009). However, studies have shown that adverse events are likely caused by mechanisms other than the inhibition of mtDNA pol-\(\gamma\). Kinetic studies have shown that AZT-TP is a poor substrate for mtDNA pol-\(\gamma\).
AZT-TP has never been detected at a concentration high enough to inhibit mtDNA pol-γ. While IC₅₀ of AZT-TP was found over 100µM, AZT was shown to inhibit thymidine phosphorylation with IC₅₀ ranging from 4.4 to 21.9µM in vitro (Lynx et al., 2006; Lynx et al., 2008). This suggests that AZT is a more potent inhibitor of thymidine phosphorylation than AZT-TP is of mtDNA pol-γ. Toxicity is likely caused by the inhibition of phosphorylating enzymes, resulting in the reduction of the intracellular TTP pools and indirect inhibition of mtDNA causing mitochondrial toxicity (Cihlar & Ray; Scruggs & Dirks Naylor, 2008).

Moreover, AZT-MP has been shown to inhibit human mtDNA pol-γ exonuclease activity in vitro. Free AZT-MP or terminally incorporated AZT binds readily in the exonuclease active site, preventing the efficient catalysis. Inhibition of mtDNA pol-γ proofreading by monophosphates and inactivation of exonuclease activity results in defective mitochondrial DNA replication, increase in mutations within mtDNA and altered mitochondrial ultrastructure (Lewis et al., 2003). In addition, it is suggested that the indirect depletion of mtDNA leads to impaired function of the electron transport chain causing an increase in production of reactive oxygen species (ROS) and oxidative damage (Modica-Napolitano, 1993; Cazzalini et al., 2001; Yamaguchi et al., 2002; Scruggs & Dirks Naylor, 2008).

In addition, it has been shown that AZT is selectively incorporated into telomeres, acting as a telomerase inhibitor and causes telomere shortening (Olivero & Poirier, 1993; Olivero et al., 1997; Gomez et al., 1998; Olivero et al., 2002; Caporaso et al., 2003; Liu et al., 2007; Zhou et al., 2007). Long-term AZT treatment is shown to induce shortening of the telomeres in HeLa cell line, in mice exposed in utero, and inhibits cell
proliferation with senescent-like phenotype induction in cultured mouse fibroblasts. Inhibition of telomerase has been proposed as the mechanism underlying AZT-induced telomeric shortening (Strahl & Blackburn, 1994; 1996; Falchetti et al., 2005). It was shown that AZT leads to intracellular accumulation of AZT-MP and inhibition of telomerase, which correlate with inhibition of cell proliferation and with AZT induced apoptotic cell death in vitro (Falchetti et al., 2005). Finally, Haik et al. showed that AZT treatment markedly resulted in a dose dependent inhibition of FGF2-induced NPC proliferation associated with a decrease of telomerase activity (Haik et al., 2000).

It is established that AZT becomes incorporated into nuclear DNA and mtDNA in place of thymidine and induces cell cycle arrest with accumulation of cells in S phase (Chandrasekaran et al., 1995; Olivero et al., 2005; Escobar et al., 2007; Olivero et al., 2008). Mechanisms involved in AZT-induced cell cycle arrest could be related to the potential of AZT to inhibit cell polymerases or to directly target proteins controlling the cell cycle and DNA repair mechanisms (Sussman et al., 1999; Escobar et al., 2007; Olivero, 2007). Indeed, it is shown that AZT induces cell cycle delay and decrease in cell proliferation in vitro with upregulation of Cyclin D1, accompanied by down regulation of Cyclin D1 associated inhibitors P18, P57, G1-S checkpoint gene P21. Moreover, Cyclin A2 was shown to downregulated in cells exposed to AZT, suggesting a block in S-G2-M progression which is consistent with the accumulation of cells in S-phase (Olivero, 2007).

The ability of AZT to incorporate preferentially into DNA increases the potential for genomic instability which may lead to formation of chromatin bridges and micronuclei (Olivero, 2007). Indeed, AZT has been shown to induce mutations, micronuclei
formation, chromosomal aberrations, sister chromatid exchange and telomeric attrition in vivo and in vitro (Gonzalez Cid & Larripa, 1994; Olivero et al., 1994; Stern et al., 1994; Ayers et al., 1996; Dertinger et al., 1996; Agarwal & Olivero, 1997; Olivero et al., 1997; Diwan et al., 1999; Sussman et al., 1999; Meng et al., 2000; Olivero et al., 2002; Poirier et al., 2003; Von Tungeln et al., 2004; Olivero, 2007). Moreover, it is reported that AZT acts as a centrosome disruptor with additional abnormalities in tubulin polymerization (Borojerdi et al., 2009).

Furthermore, in short-term in vitro incubations, AZT has been demonstrated to have anti-proliferation effects in mammalian cells (Olivero et al., 2005; Fang et al., 2009). The inhibitory effect of AZT on cell growth is shown to be associated with a combination of factors, including the induction of apoptosis, the inhibition of telomerase activity, and S and G2-M phase cell cycle arrest (Fang & Beland, 2009). It is possible that synergistic combinations of these mechanisms might exert the clinical side effects of AZT therapy.

**Distribution of AZT in the CNS**

The blood brain barrier (BBB), which is formed by cerebral blood vessels’ endothelial cells creating a barrier between the blood and the brain parenchyma with tight junctions and blood-cerebrospinal fluid (CSF) barrier (BCSFB), formed by blood vessels within choroid plexuses located in the lateral, third and fourth ventricles play an important role in distribution of AZT to the brain. It has been shown that AZT can pass through the BBB and BCSFB (Thomas & Segal, 1997; Kearney & Aweeka, 1999; Cysique et al., 2004; Evers et al., 2004; Letendre et al., 2004). However AZT’s overall penetration to the brain is limited with passive diffusion (Thomas & Segal, 1997) even though it has a small, lipophilic structure. In addition, AZT is shown to be removed from
the brain via an active probenecid-sensitive transport efflux (Dykstra et al., 1993; Takasawa et al., 1997a; Takasawa et al., 1997b). Hence, compared to the other systems the level of AZT in the CNS is low (Wu et al., 1998; Im et al., 2009). CSF to plasma concentration ratios of AZT after iv infusion have been reported in rats (0.15) (Galinsky et al., 1990), rabbits (0.26) (Wong et al., 1993), and humans 0.5 (Blum et al., 1988; Hong et al., 2001). Furthermore, Busidan et al. have shown that a single dose of radiolabeled AZT penetrated the brain very poorly, except for a notably conspicuous region of periventricular incorporation (Busidan et al., 2001). Besides, Letendre et al. classified AZT into high rank of CNS penetration –effectiveness (CPE) (rank 1) based on the chemical features, measured CSF concentrations and effectiveness of AZT in the CNS (Letendre et al., 2008).

HIV infection is associated with the disruption of BBB with an increase in the diameter of blood vessels due to inflammation of the vessel walls, alterations in the basal lamina, loss of glycoproteins in endothelial cells, endothelial cell apoptosis, and tight junction disruption (Toborek et al., 2005; Guillevin, 2008). As BBB disruption leads the HIV infected cells to enter the brain, it also facilitates the entry of drugs into the CNS (Varatharajan & Thomas, 2009).

HIV infection in the CNS leads to the development of asymptomatic neurocognitive impairment, HIV-associated mild neurocognitive disorder (MND), and AIDS dementia complex (ADC) or HIV associated dementia (HAD) with impairment in cognitive activity, memory, attention, and motor and behavioral functioning (Antinori et al., 2007). Therefore prevention of HIV infection in the CNS is one of the major goals in the field. In order to enhance levels of antiretroviral drugs including AZT in CNS and to
make them more efficient, researchers focus on developing new strategies such as developing BBB-permeable derivatives of antiretroviral drugs and efflux inhibitors, and modulating transporters (Li et al.; Saiyed et al.; Zhivkova & Stankova, 2000; Eilers et al., 2008; Miller et al., 2008; Quevedo et al., 2008; Im et al., 2009). On the other hand, the potential effects of direct exposure of excessive AZT concentrations and immune response to the toxicity on the CNS are not known.

In this study, we aimed to determine whether clinically relevant AZT regimens perturb normal levels of neurogenesis in mouse brain. We suggest that the relatively superficial location of neural stem cell niches of both SVZ and dentate gyrus with respect to the ventricles might expose these neurogenic niches to harmful levels of AZT from CSF. In addition, the cellular architecture of SVZ might expose stem cells to various signals including AZT from vascular system. Indeed, perturbed neurogenesis might play a contributing or enhancing role in neurological deficits seen in HIV (+) patients. In case of BBB-permeable AZT administration, we claim that neurogenic niches would be exposed to toxic levels of AZT leading to severe disruption of prenatal and postnatal neurogenesis.
Figure 1-1. Structural formula of AZT and Thymidine. 3' -azido-3' -deoxythymidine (AZT; C\textsubscript{10}H\textsubscript{13}N\textsubscript{5}O\textsubscript{4}) is a synthetic thymidine (C\textsubscript{10}H\textsubscript{14}N\textsubscript{2}O\textsubscript{5}) analogue in which the 3- hydroxyl group is replaced by an azido group (A).
Table 1-1. November 6, 2009, the Food and Drug Administration (FDA) approved revised pediatric dosing recommendations

<table>
<thead>
<tr>
<th>Body weight (kg)</th>
<th>Total Daily Dose</th>
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<tbody>
<tr>
<td>4 to &lt;9</td>
<td>24 mg/kg/day</td>
</tr>
<tr>
<td>≥9 to &lt;30</td>
<td>18 mg/kg/day</td>
</tr>
<tr>
<td>≥30</td>
<td>600 mg/day</td>
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CHAPTER 2
MATERIALS AND METHODS

Generation and Expansion of Astrocyte Monolayer Cell Cultures

Following decapitation, the SVZ tissue surrounding the lateral ventricles was dissected from C57BL/6 neonatal (Postnatal Day 2-4) mice brains using a sterile razor blade. The tissue was then minced and placed in ice-cold DMEM/F12 medium with N2 supplements (Gibco BRL, 17502-048), (N2 Media), containing 1X antibiotic-antimycotic (100X, Invitrogen, 15240-062) for 15 minutes. After centrifugation at 400xg for 5 minutes, the tissue was incubated in 0.25% Trypsin/EDTA solution (Atlanta Biologicals; B81310) for 5-7 minutes in 37°C water bath. Trypsin activity was inhibited by addition of N2 media containing 5% fetal bovine serum (FBS; Atlanta Biologicals). The tissue was triturated to single cell slurry by using fire-polished Pasteur pipettes. Cells were washed in N2 media and pelleted by centrifugation at 400xg for 5 minutes, and re-suspended in neural growth medium consisting of N2 media containing 5% FBS, recombinant human epidermal growth factor at a concentration of 20ng/ml (rhEGF, Sigma-Aldrich, St. Louis, MO; E9644), and basic fibroblast growth factor at a concentration of 10ng/ml (bFGF, Sigma-Aldrich, F0291). The single cell suspension was plated onto tissue culture T-25 flasks and incubated at 37°C in 5% CO2. After two days of incubation, the neural growth medium was refreshed, and cells were supplemented every other day with EGF and FGF until the primary passage of monolayer cell culture reached confluence.

Inducible Neurogenesis

Confluent primary astrocyte monolayer cells were passaged at a density of 17,500 cells/ cm² and supplemented every other day with EGF and bFGF as above for 7-10 days until confluence was established. To induce differentiation of Passage 1 astrocyte
monolayer culture, cells at a density of 17,500 cells/cm² were plated onto poly-L-ornithine (10µg/ml, Sigma, P4957) coated glass coverslips in 12-well plastic plates in neural growth medium (1ml in volume), and were supplemented every other day with 20ng/ml EGF and 10ng/ml bFGF. Four days later, the growth medium was withdrawn and replaced with an equivalent volume of N2 medium (without serum or growth factors) to induce formation of neuroblast cells. 48 hours after withdrawal of neural growth medium, cells were either fixed with 4% paraformaldehyde for subsequent immunocytochemical analysis, or quantified with a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA).

**Immunocytochemical Analysis**

Fixed cells were prepared for immunocytochemistry by washing with phosphate buffered saline (PBS) and blocking at room temperature for 30-60 minutes in PBS containing 0.01% Triton X-100 (PBSt) and 10% FBS. Primary antibodies were applied overnight in PBSt containing 10% (how many times) FBS with moderate agitation at 4°C. Residual primary antibody was removed by washing with PBS (how many times) and secondary antibodies were applied at room temperature for one hour in PBSt containing 10% FBS. Residual secondary antibodies were removed by washing with PBS. For nuclear staining, the coverslips were mounted onto glass slides and layered with Vectashield mounting medium containing 4’, 6-diamidino-2-phenylindole (DAPI) (H-1200) prior to cover-slapping. Coverslips were analyzed and photographed by using a Leica DMLB upright epifluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) with a Spot RT color CCD camera (Diagnostic Instruments). For quantification of stained cells, a minimum of 10 randomized fields were selected at 20X magnification.
Neurosphere Culture

Single cell dissociates from SVZ (Postnatal Day 2-4) were cultured in non-adherent flasks at clonal density (10,000 cells/cm²) in NeuroCult® NSC Proliferation Medium (Mouse), consisting of NeuroCult® NSC Basal Medium and NeuroCult® NSC Proliferation Supplement (Stem Cell Technologies, 05700 and 05701) supplemented with growth factors EGF and FGF at 20µg/ml and 10µg/ml respectively and heparin (2µg/ml, Stem Cell Technologies, 07980). After 7-10 days, the number and size of neurospheres (NS) were assessed and classified based on diameter (40µm, 40-80µm, >80µm) using Spot Advanced digital capture software.

Neural Colony Forming Cell (NCFC) Assay

A single cell suspension from Postnatal Day 2-4 SVZ (obtained as described above for neurosphere cultures) was plated in 35mm culture dishes at low density in a serum-free, semi-solid collagen media containing NeuroCult NCFC serum-free medium without cytokines (Stem Cell Technologies, 05720), NeuroCult Proliferation Supplement, hEGF (20µg/ml), hbFGF (10µg/ml), heparin (2µg/l) for 3 weeks. Cultures were added with Complete Replenishment Medium consisting of NSC Basal Medium, NSC Proliferation Medium, hEGF (20µg/ml), hbFGF (10µg/ml), heparin (2µg/l) once a week. By day 21-28 colonies were classified into four categories based on diameter (<0.5mm, 0.5-1mm, 1-2mm, ≥2mm) by scanning a gridded scoring dish at 4X magnification.

In Vitro Drug Treatment

AZT (TCI America, A2052) was dissolved in N2 medium, filtered through 0.22µm mesh, and stored in ready-to-use aliquots at -200C. AZT was added to the groups of cultures at 0-60µM concentration which corresponds to the range of doses administered
in human patients. Exposure times ranged from 2 hours to 48 hours, after which the medium was replaced with fresh medium without AZT. Control and treated cultures received the same number of medium changes.

**TUNEL Assay**

Apoptotic cells were labeled by using fluorimetric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (DeadEnd Fluorometric TUNEL System; Promega, G3250) according to the manufacturer's recommendations. This assay measures the fragmented DNA of apoptotic cells by incorporating fluorescein-labeled dUTP at the 3'-OH ends of DNA strands. Briefly, astrocyte monolayer cells attached onto the coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed 2x5 minutes with PBS and permeabilized by using 0.2% Triton X-100 solution in PBS for 5 minutes. After cells treatment with equilibration buffer for 10 minutes, cells were incubated within rTdT incubation buffer at 37°C for 60 minutes in a humidified chamber. Reactions were terminated by using 2X SSC for 15 minutes. After being washed 3x5 minutes with PBS, cells were counterstained with Vectashield + DAPI (H1200, Vector) and percentage of TUNEL+ apoptotic cells was calculated by assessing 10 random fields of triplicate samples.

**Senescence-Associated β-Galactosidase Labeling**

X-Gal cytochemical staining at pH 6.0 was performed as described (Dimri et al., 1995). Briefly, cells were fixed for 5 minutes in 0.2% glutaraldehyde in PBS. After two washes with PBS, cells were incubated in SAβGal staining solution containing: 1 mg/ml 5 bromo-4-chloro-3-indolyl B-Dgalactoside (X-Gal), 40mM sodium citrate pH 6.0, 5% dimethylformamide, 5% potassium ferrocyanide, 5 mM ferricyanide, 150mM sodium
chloride and 2 mM magnesium chloride for 6 hours at 37°C. Cells were washed with PBS, and counterstained with Vectashield + DAPI. The percentage of positive blue-dyed SAβGal+ cells was counted on 10 random fields of triplicate samples.

**JC-1 Assay**

The integrity of the inner mitochondrial membrane was determined by measuring the potential gradient across the mitochondrial membrane using the fluorescent stain, JC-1, as per the manufacturer’s instructions.

To determine whether AZT exposure causes changes in mitochondrial membrane potential, astrocyte monolayer cells received a 48 hour pulse of 0-60µM AZT at the time of serum and mitogen withdrawal, and mitochondrial membrane physiology was assessed via the JC-1 potentiometric dye. Control and AZT-treated astrocyte monolayer cells were suspended in warm medium at 1x10^6 cells/ml. Control cells were treated with CCCP, mitochondrial membrane disrupter, at 37°C for 5 minutes. All groups received 2µM JC-1 and were incubated at 37°C, 5% CO₂ for 30 minutes. Cells were washed and resuspended in 500ul PBS. Samples were then analyzed on a flow cytometer with 488nm excitation using the appropriate emission filter for Alexa Fluor 488 dye and R-phycoerythrin. Mitochondrial membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

**In vivo AZT Administration**

Adult, male, C57 BL/6 mice (n= 4) received daily i.p. injections of 200ul of 0.9% saline containing AZT at 0, 1, 10, 20 and 100mg/kg/day for two weeks. On the day following the last AZT injection, all animals received three BrdU injections (100mg/kg) every two hours. In order to assess immediate neurogenesis versus survival of new neurons, animals were sacrificed after two post-BrdU survival times, 1 week and 4
weeks. The brains were processed for combined BrdU/NeuN immunolabeling on a 1 in 6 series of sagittal sections, and BrdU+ cells within the dentate gyrus and SVZ were counted in a single focal plane.

**In utero AZT Administration**

C57BL/6 pregnant mice (n=4) received two subcutaneous injections of 200ul of 0.9% saline containing 0 and 250mg/kg/day AZT during the last 7 days of gestation (E12-E18; final 37% of gestation period). Pups were exposed to AZT via nursing for 3 days after birth. Astrocyte monolayers and neurospheres were generated from the SVZ of litters exposed to AZT. In addition, P3 brains were processed for paraffin embedding. Briefly brains were fixed with % paraformaldehyde and placed in embedding cassettes. After the dehydration process, the tissue was cut and mounted on glass slides to be analyzed for Ki67 immunolabeling.

**Immunohistochemistry**

The adult animals were transcardially perfused with 4% paraformaldehyde. Fixed brains were immersed in 30% sucrose for 24 hours. Using a freezing microtome, the hemispheres were cut through the sagittal plane at 40µm, and stored at -20°C in a cryoprotectant solution consisting of glycerol and polyethylene glycol.

BrdU immunolabeling was performed as previously described (Laywell *et al.*, 2005). Briefly, fixed brain sections were washed in PBS and incubated in 2xSSC:formamide (1:1) at 65°C for 2 hours. After a wash in 2xSSC, sections were incubated in 2N HCl at 37°C for 30 minutes. Finally, sections were rinsed in 0.1M borate buffer at room temperature for 10 minutes and processed for the standard immunofluorescence detection of BrdU with a rat anti-BrdU antibody (Abcam, Cambridge, MA, ab6326).
Statistics

All analyses were performed with GraphPad Prism 4.02 (San Diego, CA). Data subjected to One-way ANOVA with Tukey-Kramer or Dunnet’s Multiple Comparison Test for multiple group comparisons and unpaired T-test for two group comparisons. An asterisk (*) indicates significance (p<0.05, **p<0.01, ***p<0.001).
CHAPTER 3
IN VITRO AZIDOTHYMIDINE EXPOSURE REDUCES THE NEUROGENIC POTENTIAL OF NAÏVE SVZ STEM/PROGENITOR CELLS

Background

Azidothymidine (3’-azido-3’-deoxythymidine; AZT) is a synthetic thymidine analog in which the 3’-hydroxyl group is replaced with an azido group. Intracellularly, AZT is converted to its active form AZT-triphosphate (AZT-PPP) which competes with the natural substrate deoxythymidine triphosphate (dTTP) for incorporation by the reverse transcriptase of HIV. Once added to the growing DNA chain, AZT prevents further addition of nucleotides into the replicating strand of DNA by impeding the 5’-3’ phosphodiester linkages with its 3’-azido group. It has been shown that AZT inhibits HIV reverse transcriptase 100 times more effectively than it inhibits cellular DNA polymerase α functioning (Furman et al., 1986). AZT, as a nucleoside reverse transcriptase inhibitor, has been used in the treatment and prevention of human immunodeficiency virus-1 (HIV-1) infection alone or in combination with other antiviral agents as a part of Highly Active Antiviral Therapy (HAART). In addition, AZT monotherapy has been used in pregnancy to reduce vertical transmission of HIV-1 from mother to infant since 1994.

Although AZT’s effect is proven in controlling viral infection in adult patients and in reducing vertical viral transmission, several studies showed adverse effects of AZT such as bone marrow suppression, cardiomyopathy, hepatotoxicity, neuropathy and mitochondrial damage. Even there is extensive literature on AZT toxicity on different cell types, the possible effects of AZT administration on the neural stem and progenitor cell functioning has not been examined.

Here we investigated the hypothesis that AZT exposure perturbs neural stem and progenitor cells in vitro. We show that AZT has a strong antiproliferative effect on
cultured stem and progenitor cells. Reduced proliferation is concurrent with the onset of a senescent phenotype in AZT treated cells that alters cell morphology, differentiation potential and susceptibility to apoptosis.

Results

**Monolayer of Multipotent Astrocytic Stem Cell (MASC) Culture**

Primary MASC was generated from the SVZ tissue surrounding the lateral ventricles of C57BL/6 neonatal (Postnatal Day 2-4) mice. Confluent cell layers were then dissociated as a single cell suspension and re-plated for another series of passage to eliminate postmitotic neurons. The first passage of MASC was dissociated and plated onto poly-L-ornithine coated glass coverslips in neural growth medium, and was supplemented every other day with mitogens (EGF and FGF). Four days later, the growth medium was withdrawn and replaced with an equivalent volume of N2 medium without serum and mitogens to induce formation of neuroblast cells. 48 hours after withdrawal of neural growth medium, cells were fixed with 4% paraformaldehyde for immunofluorescence analysis with neuronal B-III-tubulin marker protein.

Here we systematically examined the time course of the growth supplement withdrawal-induced neurogenic period. In cultures which are non-withdrawn the adherent monolayer cells have a homogenous distribution (Figure 3-1 A, C). Following the first 24 hours of withdrawal of growth medium, a rapid change in monolayer cell pattern, a rosette formation consisting of B-III-tubulin (+) neuroblast cells, occurs (Figure 3-1B, D). While non-withdrawn control groups do not show any change in the number of B-III-tubulin (+) neuroblasts, withdrawn monolayer has a sharp increase of an extensive production of B-III-tubulin (+) neuroblast following the first 24 hours (Figure 3-2 A-D). We show that serum and mitogen withdrawal from second passage of monolayers leads
first to a period of relative quiescence, with little change in total cell number (Figure 3-2 C) due to both proliferation and apoptotic cell death (Figure 3-3 A-C). In addition to the baseline level of SA-B-Gal (+) cells in the control groups, serum and mitogen withdrawal causes an increase in the number of SA-B-Gal (+) cells (Figure 3-3 D).

**AZT Reduces Astrocyte Monolayer Population Expansion**

MASC culture enables us to investigate the chain of events involved in proliferation and differentiation of neurogenic stem/progenitor cells. In order to investigate the possible adverse effects of AZT on neurogenic stem/progenitor cell expansion, primary MASC were treated with 30µM AZT and analyzed for the next three passages. We show that single 7-day pulse AZT exposure of primary astrocyte monolayer cells cause a significant decrease in the expansion potential of the progeny of the next two passages. Third passage of AZT treated primary cells was able to recover its expansion potential (Figure 3-4 A). On the other hand, a single 7-day pulse of 30µM AZT exposed on the naïve first, second and third passages of MASC caused a very significant decrease in population expansion of cells. Interestingly, late passages of cells were more vulnerable to be affected by AZT (Figure 3-4B).

**AZT Abolishes Inducible Neurogenesis from Astrocyte Monolayer Cells**

As indicated above MASC can be induced to generate large numbers of neuroblasts upon withdrawal of serum and mitogens. To assess the effect of AZT on the model of inducible neurogenesis from MASC, we exposed monolayer with AZT at a clinically relevant dose range at the time of serum and mitogen withdrawal. We show that single 48 hour-pulse of 0.3-60µM AZT exposed to MASC at the time of withdrawal causes a significant decrease in both total number and %B-III-tubulin (+) neuroblasts however that effect was more severe on B-III-tubulin (+) cells (Figure 3-5 A). In order to
examine the minimum duration and dosage of AZT exposure abolishing the inducible neurogenesis, MASC was treated with 24, 8 and 2 hour-pulse of 0.03-3µM AZT at the time of serum and mitogen withdrawal. We show that even the lowest concentration of AZT, 0.03µM, exposed to cells even for only 2 hours at the time of serum and mitogen withdrawal causes a very significant decrease in the % of B-III-tubulin (+) neuroblast formation. On the other hand, MASC treated with the same concentration range of AZT for three days before supplement withdrawal did not show such a dramatic response (Figure 3-6).

**AZT Increases Apoptosis in Supplement Withdrawn MASC**

In order to investigate the possible mechanism causing AZT to abolish inducible neurogenesis, we assessed the activation of cleaved caspase-3 as an early marker in cellular apoptosis and the Terminal Uridine Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay, which detects DNA fragmentation of late-stage apoptotic cells, on MASC exposed to AZT for 48 hours applied at the time of withdrawal. We show that there is a baseline level of cells expressing caspase-3 in withdrawn control group while 30µM AZT causes a significant increase in the percentage of cells expressing caspase-3 (Figure 3-7 A). In addition, AZT exposure causes a significant increase in the percentage of TUNEL (+) cells compared to the control group (Figure 3-7 B). Both TUNEL assay and detection of cleaved caspase-3 indicate that AZT exposure causes an slight increase in early and late apoptotic events in serum and mitogen withdrawal induced astrocyte monolayers (Figure 3-7).

**AZT Upregulates SA-B-Gal Activity in Supplement-Withdrawn MASC**

Stress induced senescence, which is permanent arrest of cell division, can be induced by exposure to a variety of factors, such as UV and gamma radiation,
pharmacological agents, and oxidative stress; and is characterized by DNA damage. Senescent cells express high levels of lysosomal B-galactosidase enzyme at pH 6.0. Thus ‘senescence associated B-galactosidase’ SA-B-Gal activity has been used as a biomarker to detect senescent cells in vitro and in vivo. In order to examine if AZT, as a genotoxic agent, is causing any increase in senescent associated B-galactosidase activity, we exposed MASC with single pulse of 0.3µM and 30µM AZT for 48 hours starting at the time of serum and mitogen withdrawal. Our data show that there is a baseline level of senescent cells in controls withdrawn MASC. In addition, AZT exposure causes an increase the number of SA-B-Gal (+) cells in a concentration- and exposure time-dependent manner (Figure 3-8).

In order to understand whether AZT induced SA-B-Gal activity is due to mitochondrial damage, we performed JC-1 assay. MASCs were exposed to single pulse of 3µM and 60µM AZT following serum and mitogen withdrawal. The control cells were treated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a mitochondrial membrane disrupter, as a positive control for depolarization. 48 hours later, mitochondrial membrane physiology was assessed via the JC-1 potentiometric cationic dye which exhibits membrane potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529nm) to red (~590nm). Our data indicates that AZT exposure does not perturb mitochondrial membrane polarization of serum and mitogen withdrawn MASC (Figure 3-9).

**AZT Changes the Size and Morphology of Neurospheres**

Neurosphere assay is another in vitro cell culture system that enables us to investigate the possible effects of AZT on neurogenic stem and progenitor cells. Single cell suspensions from primary postnatal SVZ regions plated in the serum-free media
containing EGF, FGF and heparin give rise to neurospheres within 7-10 days. In order to examine whether AZT affects the expansion potential of neurosphere forming cells, one day following the single cell suspension incubation, cells are treated with a pulse of 0.3µM and 30µM AZT for 1, 3 and 10 days. At the end of exposure time, cell culture media is refreshed, and the neurosphere size and number is quantified on day 10. We show that AZT does not inhibit neurosphere formation but perturbs the formation of neurospheres Figure 3-10). Our data reveal that 1, 3, and 10 day long AZT exposure of neurosphere forming cells causes a significant reduction in neurosphere size both in concentration and exposure time-dependent manner while there is no difference in total number of neurospheres between control and AZT treated groups (Figure 3-11).

**AZT Increases the Proportion of Senescent Neurosphere Cells**

To determine whether AZT exposure causes an increase in senescence associated SA-B-Gal activity in neurosphere forming cells, we treated neurosphere forming cells with a single pulse of 30µM AZT following the day of single cell suspension incubation. Neurospheres grown in 30µM AZT were dissociated 7 days after initial cell culture and cytopspun onto glass slides and stained to detect SA-B-Gal activity. Quantification of SA-B-Gal (+) neurosphere cells reveal that AZT exposure increases the number of SA-B-Gal stained cells compared to the control group as in MASC culture (Figure 3-12).

**AZT Perturbs Formation of Neural Colonies Derived From SVZ Stem and Progenitor Cells**

Neural Colony Forming Cell Assay allows us to quantify Neural Stem Cell (NSC) and Neural Progenitor Cell (NPC) frequency in certain cell culture conditions. With high proliferative potential, NSCs form colonies ≥2mm in diameter. On the other hand,
progenitor cells which lack self-renewal ability and multipotency form colonies < 2 mm in diameter. In order to examine whether AZT affects either stem or progenitor cells or both, we exposed dissociated primary neurosphere cells, which give rise to neural colonies in certain cell culture conditions, to single pulse of 0.3µM and 30µM AZT. After 21 days in culture, colonies were classified into four categories based on the diameter. Our data show that AZT exposure causes a concentration dependent decrease in formation of neural colonies, <2mm in diameter, derived from progenitor cells but not of colonies derived from stem cells, >2mm in diameter (A-D).

Conclusion

In order to investigate possible toxic effects of AZT on neurogenic stem/progenitor cells, we employed different in vitro cell culture systems. First, we examined the effect of AZT on stem/progenitor cells of monolayers consisting of MASC. To do it, we designed an experimental paradigm which gives rise to a monolayer consisting mostly of GFP positive astrocytes but very low number of neurons and microglia. In order to reach the most homogenous monolayer, we tested different number of cells for initial seeding density of cell culture. The SVZ tissue surrounding the lateral ventricles was isolated and dissociated into single cell suspension and cultured at 17500 cells per cm² density and expanded for 7 days. By passaging the primary monolayer of MASC twice, we are able to eliminate postmitotic neurons and create more homogenous cell culture. Consistently, we always used 2nd passage of monolayers of MASC to test AZT’s effect on MASC expansion and differentiation potential. Moreover, we modified the protocol of induction of neurogenesis from monolayers of MASC. In our experimental design at the time MASCs reach 60% confluency, the growth supplements FBS, FGF and EGF are withdrawn for 48 hours to induce neurogenic differentiation from monolayers of MASC.
Here we systematically examined the time course of the growth supplement withdrawal-induced neurogenic period. In cultures which are nonwithdrawn, the adherent monolayer cells have a homogenous distribution. While the nonwithdrawn MASC has almost a linearly increasing growth curve over 7 days in culture conditions, the level of newly generated B-III-tubulin (+) neuroblasts within the monolayer is always below 5% of total population. Following the first 24 hours of withdrawal of growth supplements, a rapid change in homogenous monolayer pattern occurs. Rosette-like cell clusters become more apparent 48 hours following the withdrawal. By immunolabeling the CD45(+) microglia, GFAP (+) astrocyte, B-III-tubulin (+) neuroblast, we show that the rosette-like clusters consist of B-III-tubulin (+) neuroblasts surrounding CD45 (+) microglia (data not shown). In our induction of neurogenesis from monolayer of MASC paradigm, the level of B-III-tubulin (+) neuroblasts increases up to 45% of total population upon withdrawal of growth supplements. In contrast to the lack of growth supplements, the level of Ki-67 expressing dividing cells increases significantly within the same rosette-formation window. In addition to the cell division, the levels of TUNEL and caspase-3 (+) apoptotic cells and SA-B-Gal labeled senescent cells increase significantly within the first 48 hour of growth supplement withdrawal. Obviously, the serum and mitogen withdrawal causes significant changes in the monolayer cell population dynamics. Cell proliferation in addition to the cell death creates a turnover within the monolayer of MASC population. Different cell types seem to respond to the starvation insult in a different way however the downstream signaling mechanisms that are activated upon growth supplement withdrawal are unknown.
In order to examine if AZT affects the expansion potential of monolayer of MASCs, we performed two separate experiments. First, the single cell suspension isolated from SVZ was seeded to give rise primary monolayer of MASC. Two days after the initial seeding, the primary MASC was exposed to single pulse AZT at 30uM concentration for 7 days. In order to examine whether AZT has a long term antiproliferative effect on MASC expansion, the expansion potential of 1st, 2nd, and 3rd passages of MASC was examined. Our results show an initial decrease in expansion potential of monolayer of MASC population at 1st and 2nd passages however 3rd passage of monolayer of MASC show recovery. As a result, AZT disturbs the neurogenic astrocyte monolayer cells’ expansion potential however this effect is temporary. In the second set of experiments, we exposed 1st, 2nd, and 3rd passages of untreated, naïve monolayer of MASCs to the same concentration of AZT. In contrast to the 1st experiment results, we show a dramatic decrease in monolayer of MASC expansion potential which reduces substantially in further passages. The stem/progenitor cells residing in a quiescent state within the primary cell culture might not be affected by AZT because of the fact that actively dividing cells are more susceptible to AZT’s toxicity. As the passage number increases the number of stem/progenitor cells might decrease within the monolayer so the recovery potential of the cell population might also be reduced. In addition, it is a known fact that as the cell culture duration increases, the proliferation and survival potential of cultured cells decreases, they become senescent. Our data showing the baseline level of senescence in nonwithdrawn monolayer of MASC supports this notion.

In order to examine the potential toxic effect of AZT on the differentiation of MASC, we used our growth supplement withdrawal paradigm to induce neurogenesis. At the
time of 60% confluence of 2\textsuperscript{nd} passage of monolayer of MASC the growth supplements were withdrawn and AZT is administered to the cell culture. Following 48 hours of the withdrawal and AZT exposure, the total number of cells and B-III-tubulin expressing cells is quantified in order to represent the effect of AZT on expansion and differentiation potential of monolayer of MASC. Our data show that AZT dramatically decreases both total cell number and also B-III-tubulin expressing cell number within the monolayer. However, this effect is more severe on B-III-tubulin expressing cells. It is expected that in addition to the supplement withdrawal as a starvation insult, AZT exposure would cause a crisis within the cell culture. Even in nonwithdrawn conditions AZT causes a significant decrease in cell population expansion. Here the important detail is that AZT disturbs certain types of cells’ expansion so the levels of B-III-tubulin expressing cells could not be formed. To prove the hypothesis that AZT is selectively toxic to neuronal precursors giving rise to the B-III-tubulin expressing cells, we analyzed the minimum duration and concentration of AZT leading to the same effect. We examined the clinically relevant AZT concentrations first which is between 20 to 60UM; then we decreased the dosage up to 0.03uM. When one pulse AZT is exposed on monolayer of MASC at the time of growth supplement withdrawal for 8 hours, we show a statistically very significant reduction in the level of B-III-tubulin expressing cells within the monolayer population caused by the lowest concentration, 0.03uM, of AZT. Besides, monolayer of MASC exposed to 3uM AZT during the first 2 hours of withdrawal, results in the same response. Given the fact that the total population is not affected by the toxicity of AZT as B-III-tubulin expressing neuroblasts is, we claim that AZT specifically disturbs neurogenic cells. The decrease in total cell number of the monolayer could be
explained by disruptions of expansion potential of both neurogenic and non-neurogenic cells present in MASC population.

In our previous experiments, we showed that AZT exposure results in an increase in apoptotic cell death and senescence in control and growth supplement withdrawn monolayer of MASC. In order to investigate whether AZT exposure leads to a substantial increase in apoptotic cell death and senescent associated events within the growth supplement withdrawal induced MASC monolayer, we exposed MASC to AZT during the first 48 hours withdrawal induced differentiation. Our results show that, AZT leads a statistically significant increase in capsase-3 (+) and TUNEL (+) apoptotic cell number when exposed at relatively high concentration, 30uM. In addition, we show a statistically very significant increase in the level of SA-B-Gal labeled cells exposed to a single pulse of 0.3uM and 30uM AZT. Interestingly, compared to the control groups the level of SA-B-Gal (+) cells within the monolayer is stable within the first 24 hours of withdrawal. At the time we see a sharp increase in the B-III-tubulin expressing cell level in control conditions, AZT leads to a sharp increase in the level of SA-B-Gal labeled cells. It seems like during the first 24 hours following the withdrawal of growth supplements, neurogenic cells within the monolayer of MASC undergo a cell fate decision process during which cells become more vulnerable to the insults like AZT toxicity.

In addition to the monolayers of MASC, we examined the effect of AZT on stem/progenitor cells of neurosphere cell culture system. Neurospheres, the spherical aggregates of clonal stem/progenitor cells, are generated from single cell dissociates of SVZ isolated from neonatal mouse brain when cultured in a serum free media.
containing mitogens EGF, FGF and heparin. 10 days following the initial seeding, the individual neurospheres can be identified with smooth and well defined border. When AZT is exposed to the neurosphere forming cell culture, we see a dramatic change in size and morphology of neurospheres. Besides, our data show that AZT exposure leads to a dramatic decrease in size of neurospheres in concentration and time-dependent manner. On the other hand, this dramatic effect is not seen in neurosphere yield. Together, AZT exposure does affect the expansion potential of neurosphere forming cells however it does not kill them so that the yield stays at the same level compared to the control groups. This antiproliferative effect of AZT seems consistent with a senescence profile. In order to test if AZT upregulates senescence associated events within the neurosphere forming cell culture as seen in monolayers of MASC, we exposed neurosphere forming cells to 30uM AZT and performed SA-B-Gal labeling. Our data show that AZT significantly increases the level of SA-B-Gal labeled neurosphere forming cells consistent with monolayers of MASC data.

Moreover, we examined another in vitro cell culture system, the neural colony forming cell assay (NCFCA), in order to investigate if AZT shows a specific toxicity on stem and/or progenitor cells. NCFCA is new cell culture method which enables us to distinguish neural colonies which are derived from neural progenitor or stem cells based on their sizes. The single dissociate of primary neurospheres formed from SVZ dissociates cultured in a semi-solid collagen media including the mitogens, EGF, FGF, and heparin. In order to examine if AZT affects primary stem and/or progenitor cells to give rise neural colonies, we exposed the primary neurospheres to a single pulse of 0.3uM and 30uM AZT. AZT-treated primary neurospheres were then dissociated and
cultured in neural colony forming conditions. Our results demonstrate that 30uM AZT exposure of primary cells causes a very significant decrease in the frequency of neural colonies which are derived from neural progenitor cells but not from stem cells. Furthermore, a direct exposure of single pulse AZT at 0.3uM and 30uM concentrations leads to a very significant decrease in frequency of neural colonies which are derived from neural progenitor cells but not from stem cells. Consistent with our previous data with monolayers of MASC, the primary cells are more resistant to AZT's toxicity so colonies derived from relatively high dose AZT-exposed cells show a reduction however the direct exposure of AZT at low concentration also causes a dramatic effect in colony forming potential of progenitor cells. Interestingly, AZT has disturbed the formation of colonies derived from neural progenitors only.

Altogether, single pulse of AZT at concentrations below the clinically relevant doses leads to a dramatic decrease in expansion potential of monolayers of MASC, neurosphere forming cells, and neural colony forming cells. This effect was more severe when AZT is directly exposed to the cells. The primary cells treated with AZT show disruption in expansion potential of further progeny only when cells are exposed to relatively higher concentrations of AZT. In addition to the reduced expansion potential, our data show that AZT abolishes neural differentiation. Concurrently, we show an increase in SA-B-Gal labeling within the AZT exposed neurogenic cells. Together, we conclude that AZT induces a senescence profile in neural stem/progenitor cells in vitro. We already know that AZT is a telomerase inhibitor, causing telomere shortening. There are numerous studies showing that telomere shortening leads cells to enter a replicative senescence. However we should take the short duration of AZT exposure leading a
strong antiproliferative effect on cell expansion and abolishing effect on neural
differentiation into consideration. So we claim that telomerase inhibition by AZT
exposure is not the reason of the senescent profile. There is evidence that cellular
senescence could be induced by physiological stressor factors which can lead to
senescence more rapidly independent from telomerase inhibition and/or telomere
shortening. The DNA damage also could lead cells to enter senescence. We know that
AZT can incorporate into cellular DNA especially into mitochondrial DNA causing
mitochondrial dysfunction. In order to investigate whether AZT causes mitochondrial
dysfunction leading neurogenic cells to enter senescence, we performed JC-1 assay
however our results show that AZT does not perturb mitochondrial functioning.
Moreover, AZT is shown to lead to oxidative stress, cell cycle arrest, impairment in DNA
repair mechanisms, which are also senescent leading factors. The synergistic
combinations of these mechanisms might exert the senescent profile we observed in
our study. Future study is required to analyze the role of these mechanisms on AZT
leaded toxicity on neurogenic stem/progenitor cells in vitro.

Together, these data reveal uncharacterized effects of AZT treatment on stem and
progenitor cells. Given the fact that most human HIV (+) patients are treated with AZT
over many years, AZT regimens might perturb normal levels of neurogenesis in vivo.
Figure 3-1. Serum and mitogen withdrawal induces neurogenesis from SVZ monolayers. Multipotent astrocytic stem cells (MASC) isolated from subventricular zone (SVZ) were plated on an adhesive surface in the presence of serum and mitogens EGF and FGF. Within 7-10 days cell density become confluent (A, D). Upon withdrawal of serum and mitogens, monolayers of MASC were induced to generate large numbers of neuroblasts. 24 hours following induction by serum and mitogen withdrawal, the monolayer generated rosette-like cell clusters consisting of B-III-tubulin expressing neuroblasts (B, D). Representative phase contrast (A, B) and immunofluorescence (C, D) images are showing control (A, C) and induced (B, D) astrocyte monolayers. C, D: B-III-tubulin, red; DAPI, blue.
Figure 3-2. Serum and mitogen withdrawal leads first to a period of relative quiescence followed by a sharp increase in neurogenesis. Multipotent astrocytic stem cells (MASC) isolated from subventricular zone (SVZ) were plated on an adhesive surface in the presence of serum and mitogens EGF and FGF. 7 days later monolayer reached confluency. Panel A shows the graphical representation of MASC expansion for 7 days. At the time of 60% confluency, on day 5, serum and mitogens were withdrawn from monolayer. Quantification of immunofluorescent staining showed that 48 hours following withdrawal, the % of B-III-tubulin expressing neuroblasts within the MASC population increases dramatically (p<0.001) (B). One way Anova, Tukey's Multiple Comparison Test of significance; N=3 for all groups. Error bars represent standard deviation.
Figure 3-3. Serum and mitogen withdrawal causes a change in the monolayer cell population dynamics. At the time of 60% monolayer confluency, serum and mitogens were withdrawn from MASC culture. (A) Quantification of immunofluorescent staining showed that 48 hours following withdrawal (wd), the % of Ki-67(+) cells increased dramatically compared to the control nonwithdrawn (nwd) cells (p<0.001). The level of TUNEL (+) and Caspase-3 (+) apoptotic cells is also increased significantly (p<0.001) (B, C). SA-B-Gal staining showed that withdrawal of serum and mitogens increases the baseline level of senescence associated events compared to the nonwithdrawn control group (D). Unpaired t test significance; N=3 for all groups; *p<0.05; ***p<0.001; NS: non-significant. Error bars represent standard deviation.
Figure 3-4. AZT reduces astrocyte monolayer cell population expansion. (A) Primary MASCs were treated with 30µM AZT and analyzed for the next three passages. A single 7 day pulse AZT exposure of primary MASCs results in a significant decrease in the expansion potential of the progeny of the next two passages. Third passage of AZT treated primary cells is able to recover its expansion potential. (B) A single 7 day pulse of 30µM AZT exposed on naïve first (1stP), second (2ndP) and third (3rdP) passages of MASC causes a very significant decrease in population expansion. Late passages exposed to AZT are more vulnerable to be affected by AZT (B). Unpaired t test of significance; N=3 for all groups; *p<0.05; **p<0.01; ***p<0.001. Error bars represent standard deviation.
Figure 3-5. AZT abolishes inducible neurogenesis from monolayer cells. Astrocyte monolayer cells treated with AZT at a clinically relevant dose range at the time of serum and mitogen withdrawal for 48 hours. A single 48 hour-pulse of 0.3-60µM AZT exposed to MASC at the time of withdrawal causes a significant decrease in both total number and %B-III-tubulin (+) neuroblasts (A) however that effect is more severe on B-III-tubulin (+) cells (B). 1way ANOVA, Dunnet's Multiple Comparison Test of significance; N=3 for all groups; **p<0.01. Error bars represent standard deviation.
Figure 3-6. A short exposure of low-dose AZT perturbs inducible neurogenesis from astrocyte monolayer. MASCs were treated with 24, 8 and 2 hour-pulse of 0.03-3µM AZT at the time of serum and mitogen withdrawal (A, B, C respectively). We show that even the lowest concentration of AZT, 0.03µM, exposed to cells even for only 2 hours at the time of serum and mitogen withdrawal significantly decreases the % of B-III-tubulin (+) neuroblast formation compared to the control group (C). On the other hand, MASC treated with the same concentration range of AZT for three days before supplement withdrawal does not show such a dramatic response (D). 1way ANOVA, Dunnet's Multiple Comparison Test of significance; N=3 for all groups; *p<0.05; **p<0.01. Error bars represent standard deviation.
Figure 3-7. AZT increases apoptosis in astrocyte monolayers. MASCs were exposed to AZT for 48 hours applied at the time of withdrawal. In addition to the baseline level of caspase-3 (+) and TUNEL (+) cells in withdrawn control (A, B respectively), 30µM AZT exposure increases the percentage of caspase-3 (+) and TUNEL (+) cells significantly (A, B). One way ANOVA, Dunnett's Multiple Comparison Test of significance; N=3 for all groups; *p<0.05. NS: non-significant. Error bars represent standard deviation.
Figure 3-8. AZT upregulates senescence associated mechanisms in withdrawal induced astrocyte monolayer cells. MASCs were exposed to a single pulse of 0.3µM and 30µM AZT for 48 hours starting at the time of serum and mitogen withdrawal. Representative images show senescence-associated β-galactosidase (SAβGal) labeled cells of the control (A) and 30 µM AZT (B) exposed MASC. Quantification of SAβGal and DAPI stained nuclei (pseudocolored magenta) showed that compared to the control supplement-withdrawn cells AZT increases number of SAβGal labeled cells in a concentration- and exposure time-dependent manner (C). The baseline level of senescent cells in withdrawn control group increases markedly within the first 24 and 48 hours of AZT exposure (C). One way ANOVA, Dunnett’s Multiple Comparison Test of significance; N=3 for all groups; **p<0.01. Error bars represent standard deviation.
AZT does not perturb mitochondrial membrane depolarization in astrocyte monolayers. MASCs were exposed to single pulse of 3µM and 60µM AZT following serum and mitogen withdrawal. The control cells were treated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a mitochondrial membrane disrupter, as a positive control for depolarization. 48 hours later, mitochondrial membrane physiology was assessed via the JC-1 potentiometric cationic dye which exhibits membrane potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529nm) to red (~590nm). Compared to the non-treated supplement-withdrawn cells (A) and CCCP treated positive control groups (D), 48 hour long exposure of 3µM (B) and 60µM (C) AZT does not affect mitochondrial membrane physiology.
Figure 3-10. AZT exposure changes the size and morphology of neurospheres. Single cell suspensions from postnatal SVZ plated in a serum-free media containing EGF, FGF and heparin give rise to neurospheres within 10 days. 30μM AZT was added to the cell culture the day following the primary cells were plated. Representative phase contrast images showing differences in size and morphology of control (A) and 30μM AZT treated neurospheres (B).
Figure 3-11. AZT exposure suppresses neurosphere forming cells. Single cell suspensions from postnatal SVZ plated in the serum-free media containing EGF, FGF and heparin give rise to neurospheres within 10 days. One day following the primary cells were plated, a single pulse of 0.3 and 30µM AZT was exposed on the cell culture for 1, 3 and 10 days. At the end of exposure time, cell culture media was refreshed, and the neurosphere size and number is quantified on day 10. AZT exposure does not inhibit neurosphere formation but perturbed the formation of neurospheres. While 1 day long AZT exposure does not affect neurosphere number compared to their control levels (A), 3 and 10 day long AZT exposure on neurosphere forming cells causes a significant reduction in neurosphere size both in concentration and exposure time-dependent manner (B, C respectively). In addition, the total number of neurospheres of control and AZT treated groups is not significantly different (p>0.05) (D). One way ANOVA, Dunnett's Multiple Comparison Test of significance; N=3 for all groups; *p<0.05, **p<0.001. Error bars represent standard deviation.
AZT exposure increases the proportion of senescent neurosphere cells. One day following plating, primary cells were exposed to a single pulse of 30µM AZT. On the 7th day neurospheres were dissociated and cytopspun onto glass slides and stained to detect SA-B-Gal activity. Representative images of dissociated neurospheres with DAPI (pseudocolored magenta) and SA-B-Gal staining showing an increase in SA-B-Gal (+) cells in 30µM AZT treated neurospheres (B) compared to control group (A). Quantification of SA-B-Gal labeled cells showed that 30µM AZT exposure increases the number of SA-B-Gal stained cells compared to the control group (C). Unpaired t test; N=3 for all groups; *p<0.05. Error bars represent standard deviation.
AZT severely perturbs formation of neural colonies derived from both neural stem and progenitor cells. Dissociated primary neurosphere cell suspension was plated at a low density in semi-solid, serum-free collagen media containing growth supplements. AZT was added to the cell culture at 0.3 and 30 µM concentrations. After 21 days in culture, colonies were classified into one of four categories based on diameter. AZT treatment of SVZ stem and progenitor cells causes a concentration dependent decrease in neural colony formation. Representative images of neural colonies showing AZT exposure at 0.3 (B) and 30µM (C) concentrations yields smaller neural colonies compared to the control group (A). AZT pre-treatment of primary neurospheres does not affect formation of neural colonies (D) as severe as of colonies which were exposed to AZT directly (E). 30µM AZT pre-treatment disturbs formation of colonies smaller than 2mm in diameter, colonies derived from neural progenitor cells, but not colonies larger than 2mm which are colonies derived from stem cells (D). One way ANOVA, Dunnett's Multiple Comparison Test of significance; N=3 for all groups; *p<0.05, **p<0.001. Error bars represent standard deviation. NCFC frequency (%) = Number of colonies/total cells plated*100.
CHAPTER 4
IN VIVO AZIDOTHYMIDINE ADMINISTRATION DISTURBS NEUROGENESIS

Background

The Effect of AZT on Adult Neurogenesis

At the end of 2008, 33.4 million people worldwide were estimated to be living with human immunodeficiency virus-1 (HIV-1) infection. Besides, about 2.7 million of adults and children are newly infected and 2 million adults and children died due to AIDS. Azidothymidine (AZT) also known as Zidovudine or Retrovir is a thymidine analog and is an integral part of HAART as a nucleoside reverse transcriptase inhibitor (NRTI). It has been used in HIV treatment and prophylaxis of infection inhibits HIV-1 reverse transcriptase by acting as a competitive inhibitor of thymidine and causes proviral DNA termination. AZT is one the antiviral drugs classified into high CNS penetration (rank 1) ranking (Letendre et al., 2008). Indeed, it is shown that AZT can enter the CNS by passive diffusion across the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) Thomas & Segal, 1997; Kearney & Aweka, 1999; Cysique et al., 2004; Evers et al., 2004; Letendre et al., 2004) while it is removed from the brain via an active probenecid-sensitive transport efflux (Dykstra et al., 1993; Takasawa et al., 1997a; Takasawa et al., 1997b). More importantly, AZT is shown to incorporate into the periventricular region of the brain (Busidan et al., 2001). Surprisingly, little attention has been focused on investigating the effect of AZT exposure on the CNS.

While subventricular zone (SVZ) stem and progenitor cells are located immediately subjacent to the ependymal lining of the anterolateral wall of lateral ventricle, the mouse hippocampus is located within the posteriomedial aspect of the lateral ventricle forming a part of the posteriosuperior border of the third ventricle. In the
adult brain SVZ neural stem cells (type B) have an apical ending which directly contacts with the ventricle and a basal process ending on blood vessels (Mirzadeh et al., 2008). Moreover, it is shown that the NSCs contacting blood vessels lack astrocyte endfeed and precyte coverage (Tavazoie et al., 2008). This modified blood brain barrier structure exposes SVZ stem cells to various signals including AZT from the vascular system. In addition, the relatively superficial location of neural stem cell (NSC) niches of both SVZ and dentate gyrus with respect to the ventricular spaces makes it likely that passive diffusion of AZT from the CSF is the primary mechanism to access to neural tissue may be sufficient to expose these persistent germinal matrices to significant levels of AZT. Altogether, the present data support the hypothesis that AZT has adverse effects on both adult and perinatal neurogenesis in vivo.

In the first part of this chapter, we focused on investigating the possible toxic effects of therapeutically relevant doses of AZT exposure on cellular proliferation in the neurogenic niches SVZ and dentate gyrus of hippocampus of the adult brain. Here, we have performed two different experiments in which short term exposure of low and moderate AZT doses have been examined. In this study, young adult mice were administered 20 and 100 mg/kg/day AZT for 14 days. Neurogenesis was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation within dentate gyrus and SVZ. The quantification of BrdU (+) cells revealed that a two-week course of both low and moderate dose AZT administration does not change the number of BrdU (+) cell number in both dentate gyrus and SVZ. While the survival of dentate gyrus cells is not affected, BrdU (+) SVZ cells show a very significant decrease in mice administered with moderate dose of AZT administration.
The Effect of in utero AZT Exposure on Neurogenesis

The AIDS Clinical Trials Group Protocol 076 (ACTG 076) study established that AZT administration to pregnant women with HIV infection prenatally and during labor and to newborn infants, reduces the rate of perinatal HIV infection by about two thirds (Connor et al., 1994). In addition, when the AZT monotherapy regimen is combined with elective Cesarean Section (CS) delivery, transmission rates of about 2% were reported (1999; Mofenson, 2000). Currently, in order to prevent mother-to-child transmission of HIV-1, AZT monotherapy is administered at 14-34 weeks of pregnancy at 600mg/day, during labor at 2mg/kg loading dose followed by 1mg/kg/hour and at 8mg/kg/day dose for the neonate for 6 weeks (Witt et al., 2004). It is shown that AZT crosses the placenta rapidly with similar concentrations in maternal plasma, amniotic fluid and cord blood plasma. The AZT monotherapy regimen is shown to result in an average plasma concentration of 0.82 μg/ml in the mother and 0.75 μg/ml in the newborn (Capparelli et al., 2005).

AZT monotherapy is shown to decrease the mother to infant viral transmission rates however there are several adverse effects of perinatal AZT monotherapy have been reported. In 1999, Blanche et al. suggested that perinatal exposure of AZT may occasionally lead to mitochondrial toxicity shown by abnormality in respiratory chain complex activity, some alterations in brain morphology, neurological anomalies, cognitive and impairment, and episodes of seizures in children exposed to AZT in utero and after birth (Blanche et al., 1999; Blanche et al., 2006). In addition, it is shown that the proportion of birth defects was greater in the central nervous system (CNS), heart and chromosomes after prenatal AZT exposure in a medicaid population (Newschaffer et al., 2000). Moreover, by analyzing data from the National Institute of Child Health and
Human Development (NICHD) International Site Development Initiative Perinatal Study, Joao et al. reported CNS anomalies such as anencephaly, microcephaly, agenesis of the corpus callosum, ventricular cysts following anomalies in cardiovascular and musculoskeletal system even though the prevalence of these congenital anomalies in CNS was found relatively low (Joao et al.).

In addition to the human studies which are mostly limited with cohort studies, a number of animal studies were conducted to evaluate possible adverse effects of prenatal exposure of AZT. AZT was detected in DNA of fetal liver, lung, heart, skeletal muscle, brain, testis, and placenta in *Macaca mulatta* (Poirier et al., 1999; Slikker et al., 2000). Moreover, transplacental exposure was found to cause mitochondrial dysfunction. Alterations of oxidative phosphorylation complexes were shown in mitochondria of *Erythrocebus patas* brain, heart, and muscle (Ewings et al., 2000; Gerschenson et al., 2000; Gerschenson & Poirier, 2000). Moreover, DNA attrition was shown in monkeys and mice exposed to the drug *in utero* (Olivero et al., 1997). Furthermore, telomeric shortening was observed in tissues like brain, lung and liver of transplacentally treated mice (Olivero, 2007).

Finally, the offspring of AZT-treated rodents were shown to have neurobehavioral abnormalities such as deficits in motor responses, investigative/exploratory and social behavior, learning and spatial tasks suggesting that AZT interferes with CNS development (Petyko et al., 1997; Busidan & Dow-Edwards, 1999; Calamandrei et al., 1999a; Calamandrei et al., 1999b; Rondinini et al., 1999; Venerosi et al., 2000; Calamandrei et al., 2002a; Calamandrei et al., 2002b; Venerosi et al., 2003; Melnick et al., 2005; Venerosi et al., 2005).
In the previous chapter we reported reduced proliferation and differentiation potential of neural stem and progenitor cells following AZT treatment in vitro. In addition to findings listed above, given the fact that after a single dose of 150 mg/kg AZT administration to E20 pregnant rats, the distribution of AZT in fetus brain is heterogeneous with relatively greater amounts of AZT in the periventricular area (Busidan et al., 2001) we hypothesize that in utero administration exposes the neurogenic regions to AZT, and may disrupt prenatal and early postnatal neurogenesis.

In order to examine whether perinatal exposure of AZT perturbs prenatal and early postnatal neurogenesis, 250 mg/kg/day AZT was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. The AZT concentration and exposure period was chosen on the basis of literature reports. We show that overall pregnancy was not affected by AZT exposure so that the litter size and pup weight did not change. We showed a significant decrease in the potential of inducible neurogenesis from astrocyte monolayer cells isolated from pups treated with AZT in utero. On the other hand, the expansion potential of monolayers was not affected by AZT administration. In addition, we show altered proliferation of neurosphere forming cells giving rise to smaller neurospheres. Finally, the cellular proliferation in the neurogenic regions of the pup brain is examined. We show only a slight decrease in Ki67(+) cell number in offspring’s brains.
Results

In Vivo AZT Administration Does Not Affect Brdu (+) Cell Number in Adult Neurogenic Area

Given the information that AZT can pass through the BBB and BCSFB, and classified as rank 1 drug with high CNS penetration, we aimed to determine if in vivo administration of AZT alters the neurogenic cells’ proliferation potential. The fact that neurogenic niches SVZ and dentate gyrus of hippocampus are located relatively superficially with respect to ventricles also indicates that passive diffusion from the CSF may be sufficient to expose these persistent germinal matrices to significant levels of AZT. In addition, the subventricular zone (SVZ) stem cells (type B) have contact with both lateral ventricle and the blood vessels we claim that AZT may show its toxicity on SVZ stem cells more significantly.

In the present day, for human adults the total recommended daily amount of AZT is 600 mg/kg which is about 10mg/kg. In addition, the FDA recommended pediatric dosage is 24-600mg/kg/day. In order to equate mouse and human doses, we used mg/m² conversion factors based on FDA’s recommendation. Accordingly, 10mg/kg dose, which is 600mg AZT/day, administered to human with 1.1710m² body surface area would be equal to 133mg/kg dose administered to mouse with 0.007m² body surface area. First, we conducted an in vivo experiment where only low dose AZT administration’s effect was examined. We injected AZT at 0, 1, 10 and 20 mg/kg/day doses via intraperitoneal (i.p.) injections for two weeks. On the day following the last AZT injection, all animals received 4 BrdU injections (100mg/kg) separated by two hours. In order to assess immediate cell proliferation and survival of BrdU (+) cells, in the following 1 week and 4 weeks, the animals were sacrificed and their brains were
processed for BrdU immunolabeling in SVZ and dentate gyrus (Figure 4-1). Our results show that 2 week long, low dose administration of AZT does not change the number of BrdU (+) cell number both in dentate gyrus and SVZ (Figure 4-2).

In the second set of our experiments, we examine the effect of moderate dose of AZT administration on adult neurogenesis in vivo. In our first set of experiments the dose regimen of AZT we have tested was 67 mg/kg/day AZT for human patients, which was lower than any therapeutic dose. Here, we injected AZT at 100mg/kg/day dose, of which human equivalent dose is 335mg/kg via i.p. injections for two weeks. On the day following last AZT injection, all animals received 8 BrdU injections (50mg/kg) separated by 2 hours. Two survival times of 1 week and 4 weeks of BrdU (+) was cells examined. We show that only highly proliferative SVZ region show a significant decrease in survival of BrdU (+) cell number (Figure 4-3).

**In Utero AZT Administration Does Not Affect the Litter Size or Pup Weight**

HIV infection from mother to child, also called perinatal or vertical transmission, occurs during pregnancy, labor, delivery or breastfeeding. In 1994, the AIDS Clinical Trials Group Protocol 076 (ACTG 076) study established that AZT monotherapy plays a beneficial role in reducing mother to child transmission of HIV (Connor et al., 1994). Since then, AZT given to pregnant women infected with HIV and their newborns reduced the risk of HIV transmission. However, little is known about the impacts of this therapy on developing brain more specifically on prenatal neurogenesis.

In order to investigate whether in utero exposure of AZT perturbs prenatal and early postnatal neurogenesis, AZT at 250 mg/kg/day (~5mg/day) dosage which was chosen on the basis of literature reports, was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. There was no significant
difference in the weight and number of mouse offspring between control and AZT-treated groups (Figure 4-4 A-B). In addition, at the end of AZT administration, the weight of pregnant mice was also not altered compared to the control group Figure 4-4 C).

**In Utero AZT Administration Does Not Affect the Expansion Potential of Astrocyte Monolayer Cell Population Derived from Offspring’s SVZ Cells**

In order to investigate the effect of *in utero* treatment of AZT on neurogenic stem/progenitor cell expansion, primary MASC were generated from P3 offspring. Following decapitation, the SVZ tissue surrounding the lateral ventricles was dissected from offspring brains. The dissociated SVZ tissue was re-suspended in neural growth medium consisting of N2 media containing 5% FBS, recombinant human epidermal growth factor and basic fibroblast growth factor. The single cell suspension was plated onto tissue culture T-25 flasks and incubated at 37°C in 5% CO2. After two days of incubation, the neural growth medium was refreshed, and cells were supplemented every other day with EGF and FGF until the primary passage of monolayer cell culture reached confluence. Confluent primary astrocyte monolayer cells were passaged at a density of 17,500 cells/ cm² and supplemented every other day with EGF and bFGF for 7-10 days until confluence was established. Six passages of MASC derived from *in utero* AZT exposed offspring were analyzed and quantified with a Z2 Coulter Counter. Our results show that *in utero* AZT administration causes a decrease in the expansion potential of MASC derived from offspring’s SVZ cells only in the first passage, further passages show recovery (Figure 4-5 A).
In Utero AZT Administration Causes a Very Significant Decrease in Inducible Neurogenesis Potential of Astrocyte Monolayer Cells Derived from Offspring’s SVZ Cells

To induce differentiation of Passage 1 astrocyte monolayer culture derived from primary MASC of SVZ of in utero AZT administered offspring, cells at a density of 17,500 cells/cm² were plated onto poly-L-ornithine coated glass coverslips in 12-well plastic plates in neural growth medium, and were supplemented every other day with EGF and bFGF. Four days later, the growth medium was withdrawn and replaced with an equivalent volume of N2 medium (without serum and growth factors) to induce formation of neuroblast cells. 48 hours after withdrawal of neural growth medium, cells were fixed with 4% paraformaldehyde for B-III-tubulin immunocytochemical analysis. Our results show that in utero exposure of AZT causes a very significant decrease in inducible neurogenesis potential of astrocyte monolayer cells derived from offspring’s SVZ cells (Figure 4-5 B).

In Utero AZT Administration Yields Smaller Neurospheres than Control Group

In order to determine if in utero exposure of AZT alters the growth potential of the neurosphere forming cell progeny, we isolated SVZ cells of P3 offspring. Single cell dissociates were plated at clonal density in non-adherent conditions including EGF, FGF and heparin. After 7-10 days, the number and size of neurospheres were assessed and classified based on diameter (40µm, 40-80µm, >80µm). Neurosphere forming cells obtained from in utero AZT exposed pups yield smaller primary neurospheres with no significant difference in total neurosphere number when compared to those obtained from control offspring (Figure 4-6 A-B).
In Utero AZT Administration Causes a Slight Decrease in Ki67 (+) Cell Number in Offspring’s Brains

Given the information that in utero exposure causes incorporation of AZT into DNA, alterations in oxidative phosphorylation complexes in mitochondria, and telomeric shortening in the brains of offspring, we examined whether in utero AZT administration affects the neurogenic SVZ stem and progenitor cells causing a decrease in cell proliferation in vivo.

We injected 0 and 250 mg/kg/day AZT to C57bL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. On the day following the last AZT injection, all pups were sacrificed and their brains were processed for Ki67 immunolabeling. The quantification of Ki67(+) cells within the brains revealed that in utero AZT administration causes only a slight decrease compared to the control groups (Figure 4-7).

Conclusion

In previous chapter we show that AZT causes severe disruption on neurogenic cell expansion and differentiation leading to a senescence profile in vitro. Here we investigate if AZT administration disturbs normal levels of neurogenesis in mouse brain when applied at clinically relevant concentrations. First, we designed an experimental paradigm to model AZT administration to the adult patients. We administered AZT to young adult male mice at 0, 1, 10, and 20mg/kg/day doses via intraperitoneal (i.p.) injections for 2 weeks. In order to label dividing cells within the neurogenic regions of mice brain, all animals were given 100mg/kg BrdU injections on the day following last AZT injection. To assess the immediate/ short-term cell proliferation one group of animals was sacrificed 1 week following the last BrdU injection. In addition, another group was sacrificed 4 weeks following the last BrdU injection to examine the survival of
BrdU (+) cells. The quantification of the BrdU immunolabeling in SVZ and dentate gyrus of hippocampus show that the immediate/short-term neurogenesis is not disturbed by AZT administration. Similarly, compared to the control groups 2-week long AZT treatment at 20mg/kg/day dosage does not cause a difference in the survival of BrdU (+) cells in SVZ and dentate gyrus. Since the human data support the hypothesis that AZT exposure affects periventricular area of the brain where the neurogenic niches reside, we further analyzed our experimental paradigm. In order to equate mouse and human doses, this time we used mg/m² conversion factors based on FDA’s recommendation. Accordingly, 10mg/kg dose, which is 600mg AZT/day, administered to human with 1.1710m² body surface area would be equal to 133mg/kg dose administered to mouse with 0.007m² body surface area. In our second set of in vivo experiment, we examined the effect of clinically the most relevant dosage of AZT, 100mg/kg/day, administered for 2 weeks. As we show with low dose (20mg/kg/day) administration, AZT at 100mg/kg dosage does not cause a significant decrease in the immediate/short-term neurogenesis. On the other hand, we show statistically a very significant decrease in survival of B-III–tubulin (+) cells in SVZ but not in dentate gyrus of hippocampus. Given the fact that the animals we used in our experimental model of AZT administration were healthy animals with intact blood brain barrier it is expected to have limited penetration of AZT through the blood brain barrier to CSF to expose SVZ and dentate gyrus to harmful levels of AZT. On the other hand, HIV infection is shown to disrupt the blood brain barrier with an increase in the blood vessel diameter due to inflammation of the vessel walls, alterations in basal lamina, disrupted endothelial cell and tight junction structure. Blood brain barrier disruption leads the entry of drugs
including AZT into the central nervous system. Since our in vivo AZT administration paradigm does not reflect the HIV pathogenesis within the brain, we suggest that the decrease in the survival of BrdU (+) cells within SVZ should be considered as a very significant disruption of adult neurogenesis. Given the fact that, the enhanced entry of AZT into the central nervous system by modulating transporters, developing blood brain barrier-permeable derivatives and efflux inhibitors of AZT is one the major goals in the field, we claim that direct exposure of AZT to the neurogenic regions would cause a dramatic disruption on the neurogenesis. It remains for future studies to design experiments modeling HIV infection within the brain and modified passage of AZT through the blood brain barrier to investigate the effects of direct exposure of excessive AZT concentrations on neurogenic niches.

In addition to the adult brain, we examined whether perinatal exposure of AZT perturbs prenatal and early postnatal neurogenesis. Currently, in order to prevent mother-to-child transmission of HIV-1, AZT monotherapy is administered at 14-34 weeks of pregnancy at 600mg/day, during labor at 2mg/kg loading dose followed by 1mg/kg/hour and at 8mg/kg/day dose for the neonate for 6 weeks (Witt et al., 2004). To create an experimental model mimicking this regimen, we injected clinically relevant doses of AZT to pregnant mice from day 12 of gestation to Postnatal Day 3. According to FDA’s recommended mg/m² conversion calculations, an average women patient with 1.6 m² body surface area is given to 600mg/kg/day AZT for the last 6 months of pregnancy. Accordingly, the clinically relevant experimental dosing would be at 131.25mg/kg/day. Since a pregnant mouse give birth to 5 to 10 pups, the distribution of AZT per pup would be less amount that a human newborn. Hence we tested AZT
dosages at 250mg/kg/day and 125mg/kg/day. Since AZT can pass through the breastmilk, we continued giving AZT injections to the adult mice till postnatal 3rd day. The gestation, pup yield, mortality, and weight were analyzed. Since the group administered with 125mg/kg/day did not give birth to enough number of pups due to the lack of pregnancy, we had to eliminate the data from this group. We show that overall pregnancy was not affected by high dose AZT exposure so that the litter size and pup weight did not change. In order to investigate the effect of in utero treatment of AZT on neurogenic stem/progenitor cell expansion, primary monolayers of MASC were generated from P3 offspring SVZ and passaged six times. Our data show a significant decrease in the expansion potential of primary MASCs derived from in utero AZT administered pup brain. Consistent with our in vitro data, we show a recovery in disturbed expansion potential of primary cells in further passages. Similarly, we show a very significant decrease in differentiation potential of monolayers of MASC derived from in utero AZT administered pup SVZ. Moreover, the neurosphere forming potential of SVZ cells was also disturbed. Consistent with our in vitro data, neurosphere forming cells derived from in utero AZT exposed pup SVZ give rise to smaller neurospheres while the yield is not changed. Finally, we analyzed dividing Ki-67 (+) cells within the neurogenic regions of pup brains. Even though there is a slight decrease in the number of Ki-67 expressing cells in AZT administered pup brains, the effect was not statistically significant. Due to experimental limitations we were not able to analyze the brain section with double immunolabeling. Because of that, we cannot claim that the Ki-67(+) cell number reflects the level of dividing neurogenic cells. Altogether, we show that in utero exposure of AZT affects SVZ stem/progenitor cells’ expansion and differentiation
potential significantly as seen in our *in vitro* cell culture paradigms. Future studies are needed to examine the role of these effects on neurodevelopment and functioning. It is important to determine clinically safe and effective dosages to lead to a better health quality for HIV (+) adults, children and infants.
Figure 4-1. Representative images of Dentate Gyrus and SVZ with BrdU and NeuN immunolabeling. (A) A coronal hemisection through the adult mouse hippocampus with BrdU (green) and NeuN (red) double staining shows the BrdU (+) dividing cells in the granule cell layer of hippocampal dentate gyrus. (B) A coronal hemisection through the mouse SVZ subjacent to the ependymal lining of the lateral ventricle (LV).
Figure 4-2. 2 week-long, low-dose administration of AZT does not change the number of BrdU (+) cell number both in dentate gyrus and SVZ. Young adult male mice were administered AZT at 20mg/kg/day dosage for 2 weeks. Quantification of BrdU labeling on on a 1 in 6 series of sagittal sections in a single focal plane was performed. (A) The short term cell proliferation 1 week after the last AZT injection, both in dentate gyrus and SVZ is not changed by 20mg/kg/day AZT administration for 2 weeks. (B) The survival of cells 2 weeks after the last AZT injection is not affected by AZT. There is only a slight decrease in BrdU (+) cell number in SVZ. Unpaired t test of significance; N=4 for all groups; p>0.05. NS: non-significant. Error bars represent standard deviation.
Figure 4-3. 2 week-long moderate treatment regimen caused a significant decrease in BrdU (+) cell number only in SVZ area. Young adult male mice were administered AZT at 100mg/kg/day dosage for 2 weeks. Quantification of BrdU labeling on on a 1 in 6 series of sagittal sections in a single focal plane was performed. (A) The short term cell proliferation 1 week after the last AZT injection, both in dentate gyrus and SVZ is not changed by 100mg/kg/day AZT administration for 2 weeks. (B) The survival of dentate gyrus cells 2 weeks after the last AZT injection is not affected by AZT. There is a significant decrease in BrdU (+) cell number in SVZ. Unpaired t test of significance; N=4 for all groups; **p<0.01. NS: non-significant. Error bars represent standard deviation.
Figure 4-4. *In utero* exposure of AZT does not affect the litter size or pup weight. AZT at 250 mg/kg/day dosage was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. (A, B) There is no significant difference in the weight and number of mouse offspring between control and AZT-treated groups. (C) At the end of AZT administration, the weight of pregnant mice is also not altered compared to the control group. Unpaired t test of significance; N=4 for all groups; p>0.05. Error bars represent standard deviation.
In utero exposure of AZT on MASC derived from offspring’s SVZ cells. AZT at 250 mg/kg/day dosage was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. Primary MASC was generated from P3 offspring. Six passages of MASC derived from in utero AZT exposed offspring were quantified. Passage 1 MASCs of SVZ derived from in utero AZT administered offspring were induced to differentiate into neuroblasts by withdrawal of serum and mitogens, EGF and FGF. 48 hours following the withdrawal, number of B-III-tubulin (+) neuroblasts was quantified. (A) In utero AZT administration causes a decrease in the expansion potential of MASC only in the first passage, further passages show recovery. All other comparisons are not significantly different. (B) In utero exposure of AZT causes a very significant decrease in inducible neurogenesis potential of astrocyte monolayer cells derived from offspring’s SVZ cells. Unpaired t test; N=3 for all groups; *p<0.05, **p<0.01. Error bars represent standard deviation.
AZT at 250 mg/kg/day dosage was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. Single cell dissociates isolated from SVZ of P3 offspring were plated in nonadhesive serum free medium supplemented with EGF, FGF and heparin. After 7-10 days of initial culture, the number and size of neurospheres were quantified based on diameter. (A) Neurosphere forming cells obtained from in utero AZT exposed offspring yield smaller primary neurospheres with no significant difference in total neurosphere number (B) when compared to those obtained from control offspring. Unpaired t test; N=3 for all groups; *p<0.05. Error bars represent standard deviation.
Figure 4-7. *In utero* exposure of AZT does not affect proliferating Ki-67 (+) cell number in offspring’s brains. AZT at 250 mg/kg/day dosage was administered to C57BL/6 pregnant mice subcutaneously from day 12 of gestation to Postnatal Day 3. On the day following the last AZT injection, all pups were sacrificed and their brains were processed for Ki-67 immunolabeling. The quantification of Ki-67(+) cells within the brains revealed that *in utero* AZT administration causes only a slight decrease compared to the control group. Unpaired t test; N=4 for all groups; p>0.05. Error bars represent standard deviation.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

Since 1987 AZT has been used in the treatment and prevention HIV-1 infection, either alone or in combination with other antiviral agents. In addition, AZT monotherapy has been recommended for use in pregnancy to reduce vertical transmission of HIV-1 from mother to fetus during pregnancy, labor and delivery or breastfeeding. On the other hand, it is still classified in Pregnancy Category C of Food and Drug Administration due to the potential risks of AZT usage to the fetus in human pregnancy (Walker et al., 2007; Durand-Gasselin et al., 2008; Read et al., 2008; Foster et al., 2009). It is shown that in a nonbreastfeeding population, AZT monotherapy administered at 14-34 weeks of gestation (600mg/day), continuous intravenous infusion during labor (2mg/kg loading dose followed by 1/mg/kg/h) and 6 weeks of oral dosing to the newborn reduced the mother to child viral transmission rate from 25.5% to 8.3%.

Although AZT’s effect is proven in controlling viral infection in adult patients and in reducing vertical viral transmission, several studies showed adverse effects of AZT such as bone marrow suppression, pancytopenia, anemia, macrocytosis, cardiomyopathy, hepatic steatosis, fatal lactic acidosis, myopathy, peripheral neuropathy, distal symmetrical neuropathy, carcinogenicity (Ayers et al., 1996; Chow et al., 1997; Zhang et al., 1998; Diwan et al., 1999; Anderson et al., 2003; Lee et al., 2003; Lewis et al., 2003; Lai et al., 2004; Lewis et al., 2004; Torres et al., 2007). Some of these pathologies, e.g. myopathies, cardiomyopathy, hepatotoxicity, are shown to resolve when AZT is removed from the patient’s therapy regimen, proving these pathologies were due to AZT and were not symptoms of AIDS (Lynx & McKee, 2006).
In addition to the adverse effects seen in adults, it is shown that in utero exposure of AZT is genotoxic and mutagenic in fetal cells of humans, causing increased somatic mutations in infants and their mothers (Diwan et al., 1999; Olivero et al., 1999; Walker et al., 2007; Witt et al., 2007). Indeed, abnormality in mitochondrial respiratory chain complex activity, alterations in brain morphology, neurological anomalies, cognitive and impairment, and episodes of seizures in children exposed to AZT in utero and after birth is reported (Blanche et al., 1999; Blanche et al., 2006). In addition, the proportion of birth defects was shown to be greater in the central nervous system, heart and chromosomes after prenatal AZT exposure (Newschaffer et al., 2000). Moreover, even though with a low prevalence, congenital central nervous system anomalies such as anencephaly, microcephaly, agenesis of the corpus callosum, ventricular cysts following anomalies in cardiovascular and musculoskeletal system of were reported (Joao et al.).

Experimental animal models of the in utero AZT treatment support these findings. AZT was detected in DNA of fetal liver, lung, heart, skeletal muscle, brain, testis, and placenta in Macaca mulatta (Poirier et al., 1999; Slikker et al., 2000). Alterations of oxidative phosphorylation complexes were shown in mitochondria of Erythrocebus patas brain, heart, and muscle (Ewings et al., 2000; Gerschenson et al., 2000; Gerschenson & Poirier, 2000). DNA attrition was shown in monkeys and mice exposed to the drug in utero (Olivero et al., 1997). Furthermore, telomeric shortening was observed in tissues like brain, lung and liver of transplacentally treated mice (Olivero, 2007).

Besides, the offspring of AZT-treated rodents were shown to have neurobehavioral abnormalities such as deficits in motor responses, investigative/exploratory and social
behavior, learning and spatial tasks suggesting that AZT interferes with CNS
development (Petyko et al., 1997; Busidan & Dow-Edwards, 1999; Calamandrei et al.,
1999a; Calamandrei et al., 1999b; Rondinini et al., 1999; Venerosi et al., 2000;
Calamandrei et al., 2002a; Calamandrei et al., 2002b; Venerosi et al., 2003; Melnick et
al., 2005; Venerosi et al., 2005). Indeed, Busidan et al. have shown that after a single
dose of 150 mg/kg AZT administration to E20 pregnant rats, the distribution of AZT in
fetus brain is heterogeneous with relatively greater amounts of AZT in the
periventricular area.

It is known that AZT can pass through the BBB and BCSFB with passive diffusion
(Thomas & Segal, 1997; Kearney & Aweeka, 1999; Cysique et al., 2004; Evers et al.,
2004; Letendre et al., 2004). Even though the level of AZT in the CNS is low compared
to the other systems, it is classified into high rank of CNS penetration – effectiveness
(CPE) (rank 1) based on its chemical features, measured CSF concentrations and
effectiveness of AZT in the CNS (Wu et al., 1998; Letendre et al., 2008; Im et al., 2009).
On the other hand, the potential effects of direct exposure of excessive AZT
concentrations and immune response to the toxicity on the CNS are not known.

Even there is extensive literature on AZT toxicity on different systems and cell
types, surprisingly little attention has been focused on investigating the neurotoxic
effects of AZT administration. In this study, we focused on investigating the possible
toxic effects of AZT on neural stem and progenitor cell functioning. We claim that the
superficial location of neurogenic niches with respect to the ventricular spaces and
recently revealed cellular architecture of SVZ type B cells, which contact with both
ventricle and blood vessels forming a modified BBB, exposes neural stem cells to various signals including AZT from CSF and vascular system.

First, we investigated whether AZT exposure perturbs neural stem and progenitor cells in vitro. In order to examine the population expansion potential of neural stem and progenitor cells isolated from naive mouse SVZ, we exposed multipotent astrocyte monolayer cells (MASC) to clinically relevant AZT concentrations. We show that AZT has a strong antiproliferative effect on MASCs. Interestingly, while treated primary cells were more resistant showing a recovery, the late passages of cells were more vulnerable to AZT. In addition to the impaired expansion potential, we examined whether AZT leads to a disruption on inducible neurogenesis from MASC. We exposed monolayer with AZT at the time of induction stimulus, which is withdrawal of serum and mitogen withdrawal. We show that even the lowest concentration of AZT we examined, 0.03µM, exposed to cells even for only 2 hours at the time of serum and mitogen withdrawal causes a significant decrease in both expansion of population and differentiation to neuroblasts however that effect was more severe on B-III-tubulin (+) neuroblasts. On the other hand, MASC treated with the same concentration range of AZT for three days before supplement withdrawal did not show such a dramatic response. In order to investigate the possible mechanism causing AZT to impair the expansion potential and to abolish inducible neurogenesis, we assessed the activation of cleaved caspase-3 as an early marker in cellular apoptosis and the Terminal Uridine Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay, which detects DNA fragmentation of late-stage apoptotic cells, on MASC exposed to AZT for 48 hours applied at the time of withdrawal. We show that there is already a baseline level of cells
expressing caspase-3 in withdrawn control group while 30µM AZT causes a significant increase in the percentage of cells expressing caspase-3. In addition, AZT exposure causes significant increase in the percentage of TUNEL (+) cells.

Stress induced senescence, which is permanent arrest of cell division, can be induced by exposure to a variety of factors, such as UV and gamma radiation, pharmacological agents, and oxidative stress; and is characterized by DNA damage. In order to examine if AZT, as a genotoxic agent, is causing any increase in senescent associated B-galactosidase activity, we exposed MASC with single pulse of 0.3µM and 30µM AZT for 48 hours starting at the time of serum and mitogen withdrawal. Our data show that in addition to the baseline level of senescent cells in control group due to the cell culture conditions, AZT exposure causes an increase the number of SA-B-Gal (+) cells in a concentration- and exposure time-dependent manner. Since DNA damage at any location including the mitochondria might lead to cellular senescence, we performed JC-1 assay to understand whether AZT induced SA-B-Gal activity is due to mitochondrial damage. However, our results show that AZT exposure does not perturb mitochondrial membrane polarization of serum and mitogen withdrawn MASC.

We also conducted experiments examining the effect of AZT on neurosphere cell culture system. Consistent with previous findings with astrocyte monolayers, AZT exposure reduces the size of neurospheres, alters their morphology, and increases the proportion of senescent neurosphere cells. Moreover, to examine the effect of AZT on stem and progenitor cells, neural colony forming cell assay (NCFCA) was used. We show AZT severely perturbs formation of neural colonies formed by both neural stem and progenitor cells isolated from SVZ.
Our results show that reduced expansion of stem progenitor cells is concurrent with the onset of a senescent phenotype in AZT treated cells that alters cell differentiation potential and susceptibility to apoptosis suggesting that AZT inhibits differentiation at the point of mitotic expansion, most likely through inhibition of early S phase of and subsequent differentiation processes. Here we show AZT exposure results in a striking inhibition of stem/progenitor cell population expansion and withdrawal induced differentiation in a dose dependent manner. The rapid blockage of differentiation by AZT suggests a direct toxic effect rather than a telomerase inhibition which is thought to be one of the main mechanisms of AZT toxicity. However the reduction in AZT exposed neurosphere diameter and MASC proliferation could be explained by perturbations in nucleoside phosphorylation kinetics, telomerase inhibition, or mitochondrial dysfunction. Here, we show that there was no disruption of mitochondrial function by AZT exposure. Overall, the adverse effect of AZT leading to a cellular senescence may be related with synergistic interaction of several toxicity mechanisms.

To examine whether *in vivo* exposure of AZT would cause any disruption on adult neurogenesis, we injected adult animals with AZT at clinically relevant low and moderate concentrations for a short term period. Neurogenesis was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation within dentate gyrus and SVZ. The quantification of BrdU (+) cells revealed that a two-week course of both low and moderate dose AZT administration does not change the number of BrdU (+) cell number in both dentate gyrus and SVZ. While the survival of dentate gyrus cells is not affected, BrdU (+) SVZ cells show a very significant decrease in mice administered with
moderate dose of AZT administration. Here we did not see a strong antiproliferative effect of AZT as seen in cell culture systems *in vitro*. If AZT exerts a direct toxic effect, we expect to see significant differences in short term neurogenesis. However, we did not observe such a difference between BrdU (+) cell of control and AZT treated groups. Similarly, long term survival was not affected. The intact structure of the brain and blood brain barriers of adult animals might prevent the passage of AZT through the neurogenic niches.

On the other hand, HIV infection is associated with the disruption of BBB with an increase in the diameter of blood vessels due to inflammation of the vessel walls, alterations in the basal lamina, loss of glycoproteins in endothelial cells, endothelial cell apoptosis, and tight junction disruption (Toborek *et al.*, 2005; Guillevin, 2008). As BBB disruption leads the HIV infected cells to enter the brain, it also facilitates the entry of drugs into the CNS (Varatharajan & Thomas, 2009). HIV infection in the CNS leads to the development of asymptomatic neurocognitive impairment, HIV-associated mild neurocognitive disorder (MND), and AIDS dementia complex (ADC) or HIV associated dementia (HAD) with impairment in cognitive activity, memory, attention, and motor and behavioral functioning (Antinori *et al.*, 2007). Therefore prevention of HIV infection in the CNS is one of the major goals in the field. In order to enhance levels of antiretroviral drugs including AZT in CNS and to make them more efficient, researchers focus on developing new strategies such as developing BBB-permeable derivatives of antiretroviral drugs and efflux inhibitors, and modulating transporters (Li *et al*.; Saiyed *et al*.; Zhivkova & Stankova, 2000; Eilers *et al*., 2008; Miller *et al*., 2008; Quevedo *et al*., 2008; Im *et al*., 2009). We suggest that in case of increased delivery of AZT into the
brain, a direct exposure to the CNS would cause more dramatic changes as we have shown with MASC, NS, NCFCA cell culture systems.

Finally, we examined whether perinatal exposure of AZT perturbs prenatal and early postnatal neurogenesis, the C57BL/6 pregnant mice were treated with 250 mg/kg/day AZT subcutaneously from day 12 of gestation to Postnatal Day 3. The AZT concentration and exposure period was chosen on the basis of literature reports. We show that the concentration of AZT was not so toxic that overall pregnancy was affected; the litter size and pup weight did not change. We showed a significant decrease in the potential of inducible neurogenesis from astrocyte monolayer cells isolated from pups treated with AZT in utero. Except the first passage of MASC, the expansion potential of monolayers was not affected. The decrease in the first passage of MASC was recovered in further passages. In addition, we show altered proliferation of neurosphere forming cells giving rise to smaller neurospheres as seen in our in vitro findings. Since the neurosphere forming cells were passaged for only once, we cannot claim that this decrease would be recovered as in MASCs. Finally, the cellular proliferation in the neurogenic regions of the pup brain is examined. We show only a slight decrease in Ki67(+) cell number in offspring’s brains suggesting that short term neurogenesis was not affected directly by AZT toxicity.

Summary

Together, these data reveal uncharacterized negative consequences of AZT treatment on neural stem and progenitor cells. Most of the toxic effects of AZT occur after long time scales, beyond the capability of our in vivo experiments with short term treatment models. The long-term use of AZT as a part of anti-HIV therapy might affect the stem and progenitor cells within the adult brain. Given the fact that HIV infection
leads to development of neurological deficits and that human HIV (+) patients are treated with AZT over years, it is important to determine what extent AZT regimens might perturb normal levels of neurogenesis to exacerbate or contribute to these neurological problems. We expect our results will reveal new insights regarding the effect of AZT on stem/progenitor cell functioning, and the development of new treatment approaches to prevent the HIV infection in CNS.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Meryem Demir was born in 1978 in Trabzon, Turkey. She graduated from Bahcelievler Anadolu High School in 1996 in Istanbul. Following graduation, she attended Istanbul University, Cerrahpasa Medical School in Istanbul, Turkey and obtained a B.S. degree in Biomedical Sciences in May 2000. She enrolled in the Institute of Biomedical Engineering at Bogazici University - Istanbul in September 2000 and graduated with a MSc. degree in September 2003. In August 2004, she entered the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida College of Medicine, leading to the degree for Doctor of Philosophy. In May 2005, Meryem joined the laboratory of Eric D. Laywell. During her graduate study, she worked on characterizing the effects of Azidothymidine on adult and perinatal neurogenesis. Upon completion of her Ph.D. in December 2010, Meryem plans to pursue a career dedicated to the study of stem cell biology.