Comparative Neurotoxicities of Amphotericin B and Amphotericin B Methyl Ester in Mice and on Oligodendrocytes in culture

By

Oluchukwu U. Nnodi

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

And

The Graduate School of Biomedical Sciences

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Joint Graduate Program in Toxicology

Written under the direction of

Professor Kenneth R. Reuhl

And approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

October 2014

ABSTRACT OF THE DISSERTATION

Comparative Neurotoxicity of Amphotericin B and Amphotericin B Methyl Ester in Mice and on Oligodendrocytes in Culture

by OLUCHUKWU UWAKWE NNODI

Dissertation Director:

Professor Kenneth R. Reuhl

Amphotericin B methyl ester (AME) similar to Amphotericin B (AMB), is a macrolide antibiotic highly effective against complex systemic fungal infections. Clinical data suggested AME was neurotoxic, but subsequent analysis of lots used in its only clinical-trial revealed contamination with AMB and multi-methylated AMB-derivatives. Accruing evidence suggests AMB not highly-purified AME causes neurotoxicity, thus, it is likely that AMB contributed to neurotoxicity observed during the AME trials. Preliminary assessment of adult Balb/c mice given alternate day intraperitoneal (i.p) AME to mimic the human dose used (5 mg/kg of body weight [b.w]) compared with similarly treated AMB mice was carried out after 1-month and 2-months of injections. Light microscopy of cerebral white matter stained with hematoxylin/eosin and luxol fast blue suggested AME caused less demyelination than AMB. AMB induced CNS demyelination persisted after 3-months of drug-cessation following 2-months of

injections. The nuclear area factor (NAF) analysis of AMB-brains provides preliminary data that AMB may cause apoptotic change in cerebral white matter of mice. Myelin lesions were absent in AME-brains, and even after 5-months of day i.p treatment of adult mice with 7-mg/kg b.w of AME alone, ultrastructural myelin changes were minimal. Differentiated human oligodendrocyte cell line (MO3.13) exposed to 0.5-30µg/ml AMB or AME for 24 hours demonstrated a progressive increase in cytotoxicity as determined by dye exclusion. The lowest concentration of AMB to induce cell injury was 1µg/ml, whereas injury first appeared in AME-exposed cells at10 µg/ml, a concentration far exceeding those expected in clinical use. AMB at1µg/ml showed apoptotic nuclear change in cells colocalizing for propidium iodide and fluorescein diacetate. Confirmation with Hoechst and fluorescent inhibition of caspases labeling revealed a significant decrease in the NAF and an increase in caspase-3 and -9 activity, suggesting induction of the intrinsic mitochondrial apoptotic pathway. AMB also caused increased cytochrome *c* release with a significant loss of mitochondrial membrane potential ($\Delta\Psi_M$) that was refractory to rescue with Trolox as determined with JC-1 dye. Cytotoxicity was not observed in similarly treated AME-cells. Our data suggests that AMB, but not AME may induce oligodendrocytes cytotoxicity and mitochondrial damage, eventually diminishing $\Delta\Psi_M$ with consequent apoptosis.

DEDICATIONS

I dedicate my dissertation work to the memory of Carl Schaffner, Professor Emeritus; your co-discovery of AME began the journey of this work, your passion drove the success. Thank you for your unwavering confidence in me, and thank you for being my friend.

To Chiedu, Chiaka, and Ugochi Nnodi; Nesoch and Okechi Awujo; Chinedu and Ebele Mbanugo; Ijeoma and Chineme Chukuezi; Dr. Sylvester and Christiana Enworom; Kelechi and Uloma Mbata, I am blessed for having you as family.

To my friends, Clement Anyadiegwu, Dr. Obi Onowu, Priscilla Ogigai, Emma Obinwa, Femi Odeleye and kene Iloenyosi, you are amazing gifts. To Patti Reuhl, it's an honor having you as a friend. To my Feline Oreo, and my Canines, Farley and Jessy, for being the most adorable friends.

To the loving memory of my dad, Anukem (Chief A.O Nnodi); thank you for the unfettered access to your microscopes et al at a tender age. Chizomam, my loving mum, thank you nurturing my gifts and talents. As a team, you spared no cost, making sure I got the best education. You will forever be my Heroes.

ACKNOWLEDGEMENTS

To Dr. Kenneth Reuhl, thank you for your patience, guidance, mentorship and friendship through these past years. Thank you for sharing your wealth knowledge. This work would not have happened without you. I'm most grateful!

To Drs. Mark Robson, Keith Cooper, Jason Richardson and Don Borders, thank you for being my role models. Most of all, I appreciate your time, expertise and the sacrifice you made to serve on my committee. You are highly valued.

To Dr. Bob Snyder (Professor Emeritus); my mentor, my teacher, my friend.

To Dr. Herb Lowndes, thank you for your confidence in me, I feel honored that I can call you my friend.

To the Gordon Road Family, Dr. Mike Iba, Dr. Maryanne Poulunas, Kathy Ellinski, Blair Weig and Dr Sung Ji, thank you for your friendship and support. We've shared so many special moments that I will carry along with me.

To Sandi Selby, Liz Rossi, Bernadine Chmielowicz, Rosemary Hayes, Liza Pek and BJ Coram. I feel special having you as my friends, your support and assistance as colleagues cannot be measured. Thank you for being my resource.

Drs. Janet Gould and Aileen Hayes, you will forever have a place in my heart for all the skills you deposited in me. I feel privileged to have been considered worthy to work with you at Bristol Myers Squibb.

To the JGPT Faculty, too numerous to mention and my fellow RATS, thank you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

ABLC	amphotericin B lipid complex
AFIP	Armed Forces Institute of Pathology
AMB	amphotericin B
AME	amphotericin methyl ester
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
AP	action potential
ApaF	apoptosis protease-activating factor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BBB	blood brain barrier
b.w	body weight
^{o}C	Celcius
C. albicans	Candida albicans
C. posadasii	Coccidioides posadasii
C.immitis	Coccidioides immitis
CCCP	carbonylcyanide m-chlorophenylhydrazone
CDC	Center for Disease Control
CM	coccidioidal meningitis
CNS	central nervous system
CO2	carbon dioxide
COX	cytochrome oxidase
CsA	cyclosporine A
CSF	cerebro spinal fluid
Cyp	cyclophilin
Cyt c	cytochrome c
DAB	diamino benzidine
D-AMB	deoxycholate Amphoterin B (Fungizone®)
DEVD	aspartate-glutamate-valine-aspartate
DM-20	proteolipid protein isoform
DMEM	Dulbecco's 'eagles' medium
DMSO	dimethyl sulphoxide
DNA	deoxynucleic acid

DPPC	dipalmitoyl phosphatidyl choline
EM	electron microscopy
FAM	carboxyfluorescein
FDA	Food and drug administration
FDA	fluorescein diacetate
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLICA	fluorochrome inhibition of caspases
FMK	fluoromethyl ketone
g	gram
GalC	galactosylceramides
GFAP	glia fibrilary acid protein
GFR	glomerular filtration rate
GluR	glutamate receptor
^{3}H	tritium
H/E	hematoxylin and eosin
HCl	hydrochloric acid
HIV	human immuno deficiency virus
HPLC	high performance liquid chromatography
hrs	hours
i.p	intraperitoneal
i.t	intrathecal
i.v	intravenous
IFN	interferon
IgG	immunoglobulin
JC-1	5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzamidazoleocarbocyanin iodide
kDa	kilodalton
kg	kilogram
L	liter
LC50	lethal concentration 50%
LC10	lethal concentration 10%
LDL	low density lipoprotein
LEHD	leucine-glutamine-histidine-aspartate
LETD	leucine-glutamine-threonine-aspartate
LFB	luxol fast blue
LIF	leukocyte inhibitory factor
mg	milligram
Min	minutes
ml	milliliter
mM	millimole

MOG	myelin oligodendroglia glycoprotein
MPT	mitochondrial permeability transition
mRNA	messenger ribonucleic acid
MW	molecular weight
nm	nanometer
NO	nitric oxide
PBR	peripheral benzodiazepin receptor
PBS	phosphate buffered saline
PDGFR	platelet derived growth factor receptor
PE	phosphatidyl ethanolamine
PI	propidium iodide
PLP	proteolipid protein
PNS	peripheral nervous system
PVL	peri-ventricular
RD	rhabdomysarcoma
ROS	reactive oxygen species
RT	room temperature
S. serevisea	*Sacromyces cerevisea*
SE	standard error
SFM	serum free media
Spp	species
SR	sulphur rhodamine
SSKI	saturated solution of potassium iodide
SVZ	sub ventricular zone
t½	half-life
T3	tyroxine
TEA	tetraethyl ammonium
TNF	tissue necrosis factor
µ	micron
µg	microgram
UV	ultraviolet
$\Delta\Psi_m$	mitochondrial permeability transition event
Vd	volume of distribution
VDAC	voltage dependent anion channel
WHO	World Health Organization
wt/wt	weight for weight
Z	amino terminal benzoylcarbonyl group

Chapter 1

1 Introduction and Literature Review

1.1 Background

There is a serious need for improved antifungal therapies. Many fungal infections, particularly disseminated fungal infections, such as fungal meningitis, remain extremely difficult to manage clinically, and are usually accompanied by significant morbidity and mortality (Mathisen et al., 2010). Disseminated fungal infections are prevalent in immunocompromised patients, a population that is expanding rapidly in the Third World. Further, global population movements are enhancing exposure to fungal species previously restricted in range (Warnock, 2007). In addition, climate change that leads to alternating long periods of drought following brief spells of rainfall significantly enhance the spread of potentially lethal species of fungi such as *Coccidioides immitis,* the etiological agent of valley fever and coccidioidal meningitis in the United States. The rains allow the spores to bloom in the soil, while arid conditions escalate the airborne transmission of the fungal-spores (Galgiani et al., 2005).

As the incidence of invasive fungal infections has continued to increase over the past three decades, so has their importance as a cause of morbidity and mortality worldwide. This problem is particularly severe in the developing world, where poverty,

poor sanitation, social upheaval and epidemic levels of immunosuppressive diseases like HIV are ubiquitous (Richardson, 2005). However, fungal infections are by no means restricted to the Third World, but are also common in countries whose sophisticated medical care supports patients at enhanced risk: patients with solid organ or stem cell transplants and patients following cancer chemotherapy. In addition, patients with in-dwelling intravascular lines or urinary catheters, prolonged broad-spectrum antibiotic use and cases of burns or severe systemic illness are likewise at significantly elevated risk (Clark and Hajjeh, 2002; Richardson, 2005).

Despite the rising incidence of fungal disease worldwide, the introduction of new antifungal drugs is failing to keep pace (Sangamwar et al., 2008). It may be because drug development is an intricate process that is risky, expensive and time consuming (DiMasi, 2001; DiMasi et al., 2003). Perhaps this is why only few new classes of effective antifungals were developed over the past 60 years (Table 1.1). In addition, fungal cells closely resemble mammalian cells, presenting a major clinical challenge in designing new drugs that selectively target the fungi without toxicity to the host (Georgopapadakou and Walsh, 1994).

Host toxicity is generally an accepted risk with existing antifungal therapies (Debono and Gordee, 1994). This is especially true for Amphotericin B (AMB), which has been the choice for treatment of life-threatening fungal infections for almost six decades. Use of AMB is complicated by significant side effects (Winn, 1979; Racis et al., 1990; Reuhl et al., 1993; Bratjburg and Bolard, 1996). However, notwithstanding these limitations and the introduction of newer antifungal agents, AMB remains the most effective and widely used antifungal agent worldwide.

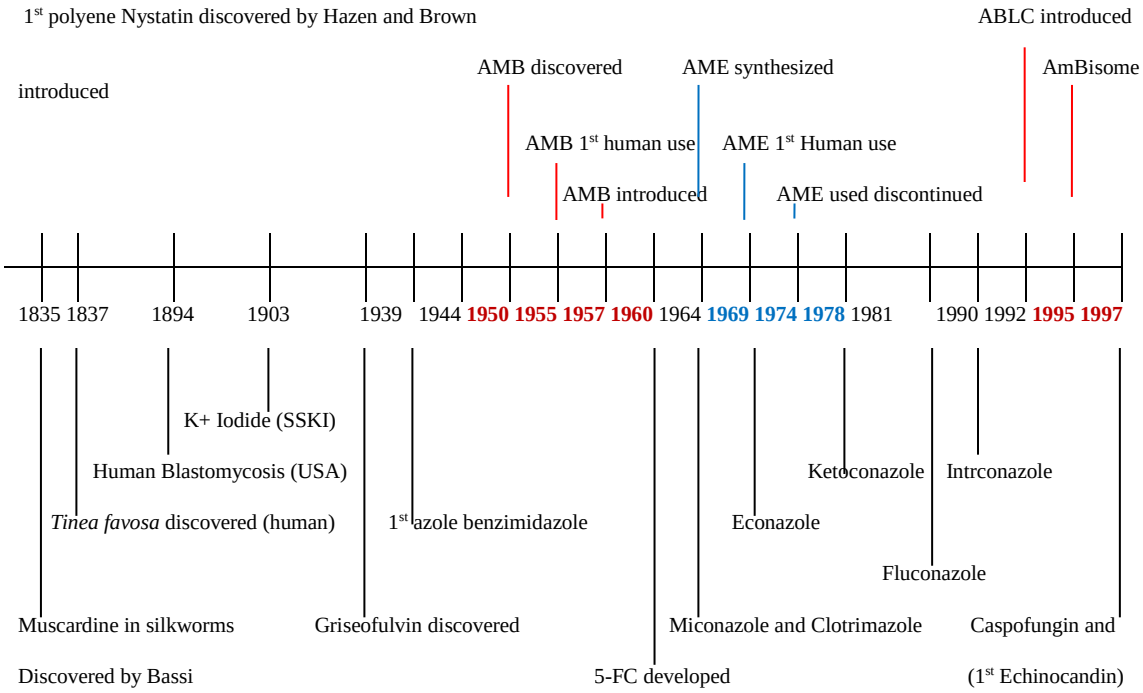
Mechlinski and Schaffner (1972) modified the AMB molecule in attempt to overcome these safety concerns and to improve AMB's therapeutic efficacy. This led to the development of Amphotericin B methyl ester (AME). AME is a semi-synthetic derivative of AMB, which is water-soluble (Schaffner and Mechlinski, 1972), with a considerably reduced renal toxicity and excellent antifungal activity (Keim et al., 1973; 1976; Hoeprich et al., 1988). Nevertheless, economic and logistical constraints have affected adequate research to understand the molecular mechanism of AME's antifungal action, knowledge of which is critical to its acceptance for clinical use. This dissertation examines the effects of AME exposure on brain myelin and oligodendrocyte cell lines and benchmarks AME against its parent antibiotic, AMB, with the aim of unraveling their mode of action and establishing limits for AME's use.

1.1.2 Coccidioidomycosis (Valley Fever)

Coccidioidomycosis, also termed valley fever is a highly infectious fungal disease of increasing global concern that has humans and animals such as dogs as important hosts. The dimorphic fungi, *Coccidioides immitis/posadasii* (Stevens, 1995) causes the disease. It is found in arid and semi-arid regions, particularly in soils of the southwestern US (Arizona, California, New Mexico), and parts of Central and South America (DiCaudo, 2006). Coccidioidomycosis has always been endemic to the southwest US; hence its other name, San Joaquin Valley fever. Fungal spores (athraconidia) carried by the wind from soil in endemic areas, usually initiate infection in humans via inhalation.

Figure 1.1 History of antifungal development and agents approved for systemic mycosis in the US.

(Modified from Gubbins and Anaissie, 2009)



These transmission modes present a public health risk because of the potential infection of large populations during dust storms (Galgiani et al., 2005).

Although coccidioidomycosis infection usually causes a mild asymptomatic to moderate illness, it can also lead to a severe disseminated disease. CNS involvement is of greatest concern (Dixon, 2001; Chiller et al., 2003; Deresinski, 2003; Arsura et al., 2005), with cerebro-meningeal coccidioidal infection (CM) occurring in a third to about half of patients presenting with disseminated disease (Buñuelos et al., 1996). Inflammation of the meninges is common, with encephalitis and vasculitis with eventually progressing to brain abscess, hydrocephalus, hemorrhage and stroke (Ritter et al., 2013). The complexity of neural infection may confuse adequate clinic-pathologic diagnosis.

Without treatment, most patients with CM will die within weeks to months. This is especially true in the Third World where access to medicine is deficient. A fatality rate of close to 100% (Johnson and Einstein, 2006), with a mean survival of thirteen months was associated with CM in the era pre-dating the availability of treatment (Vincent et al., 1993). AMB given i.v and intrathecally/ intracisternally was the earliest efficacious treatment for CM (Galgiani et al., 2005). Mortality values for CM improved with AMB treatment to 49% (Vincent et al., 1993). The introduction of oral azoles such as fluconazole and Ketoconazole to the therapeutic armamentarium brought further improvement (Jonson and Einstein, 2006). However, both AMB and the azoles are associated with significant toxicity. Despite the troubling toxicities of these antifungal agents, allowing cerebro-meningeal coccidioidal infection (CM) to go untreated is not an option.

1.1.3 AMB Use in Coccidioidomycosis and its Limitations

The discovery of the polyene macrolide AMB (Figure 1.1) at the Squibb Institute and isolation from the actinomycetes *Streptomyces nodusus* (Waksman, 1965; 1962; Dutcher, 1968; Utz, 1980) by Stiller, Vandeputte and Wachtel (1955) marked a turning point in the treatment of systemic fungal infections (Einstein et al., 1961). Particularly, it is the first, and until relatively recently the sole parenteral agent to induce significant remission of CM (Winn, 1958; Einstein et al., 1961). However, AMB is a well-known cause of morbidity and profound toxicity that restricts its use, earning it the nickname "amphoterrible" amongst clinicians.

Why is AMB so toxic to humans? The answer may lie in its unique physical, pharmacologic and pharmacokinetic properties. AMB, as with polyenes in general, are amphipathic (Figure 1.2). A common mechanism amongst them is the formation of membrane ion channels (Figure 1.3) facilitated by the hydrophobic domain interaction with lipids in membranes (Kinsky, 1966; Hamilton-Miller, 1973; Bratjburg and Bolard, 1996). There is abundant scientific evidence to support the theory that formation of membrane pores occurs by the interaction of AMB with membrane sterols leading to efflux of monovalent cations, particularly K^+ (Figure 1.3), and subsequent cell death (Gottlieb et al., 1958; Kinsky, 1966, Hamdan and Resende, 1988; Zumbuehl et al., 2004). AMB-induced cell death can be blocked by TEA (tetraethyl ammonium), a potassium channel inhibitor (Brajtburg et al., 1980).

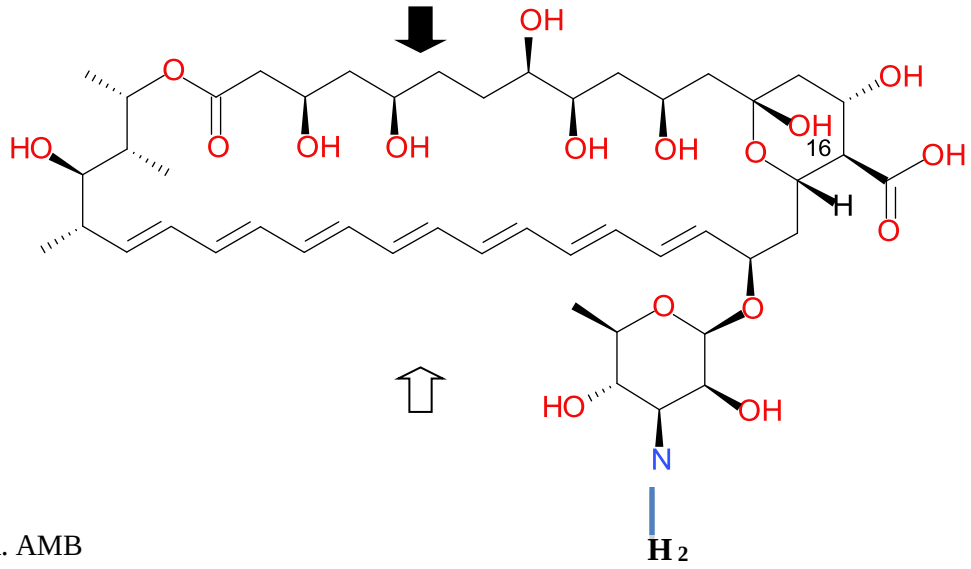
In addition, both fungi and humans are eukaryotes, possessing relatively similar membrane sterols (Figure 1.4) that can interact with AMB. However, the relative

selective interaction of AMB with ergosterol, the unique sterol found in fungal membranes (Fisher et al., 1969; 1975; 1978; 1979) forms the important basis for its therapeutic action on fungal pathogens (Gale, 1973; Hospenthal et al., 1989). Unfortunately, a lesser capacity to interact with mammalian membrane sterol, cholesterol can cause considerable toxicity to the host (Bonner et al., 1976).

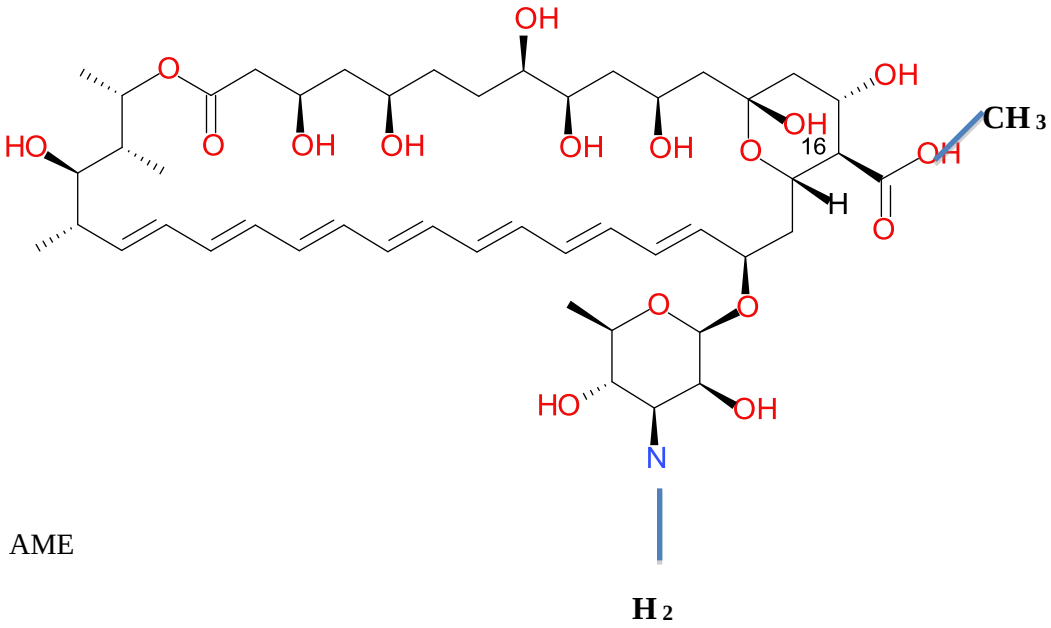
AMB's insolubility in water is related to its toxicity (Mazerski et al., 1990; Bratjburg and Bolard, 1996). The carboxyl and amino groups of AMB are simultaneously charged at neutral pH, making the AMB molecule amphoteric. The fact that AMB is amphoteric makes it a zwitterion, and thus it has a net charge of zero at neutral pH. This property, along with its intrinsic ability to form intra-molecular self-aggregates (colloids), contributes to it poor aqueous solubility (Bratjburg and Bolard, 1996). These aggregates are associated with increased AMB toxicity and appear to form in a pH-dependent manner. The monomeric form tends to form at pH >10 imparting AMB with a net negative charge. Conversely, at pH below 10 the aggregates tend to form and AMB acquires a net positive charge (Romanini et al., 1999).

Furthermore, AMB's poor aqueous solubility leads to poor membrane permeation, restricting its gastrointestinal (GI) absorption and limiting oral bioavailability (Lemke et al., 2005). Fortunately, solubilization of AMB is possible (Schaffner and Mechlinski, 1972). Coupling AMB with the salts of bile acids (detergent) such as sodium deoxycholate (Fungizone®), or more recently with lipids, is achieved without loss of activity, allowing it a water-dispersion fit for parenteral administration (Einstein et al.,

Figure 1.2 Representative structures of Amphotericin B and its methyl ester analog. (A) Amphotericin B, black arrow depicts the hydrophilic chain and white arrows indicate the hydrophobic chain. (B) Amphotericin B methyl ester, the methyl ester derivative, is less toxic. (Structures drawn using Symyx draw 3.2, Symyx Technology Inc. US)



A. AMB



B. AME

Figure 1.3 Therapeutic and toxic mechanism of AMB/AME

(a) Amphotericin B is believed to assemble into a trans-membrane channel. (b) Synthetic conversion of the C41 carboxylic acid of Amphotericin B into the corresponding methyl fragment was found to fully preserve antifungal activity of the resulting chemical probe. AMB, like AME, binds the sterol membrane of target cells. This interaction induces membrane channel permeability with subsequently leakage of cellular contents and event

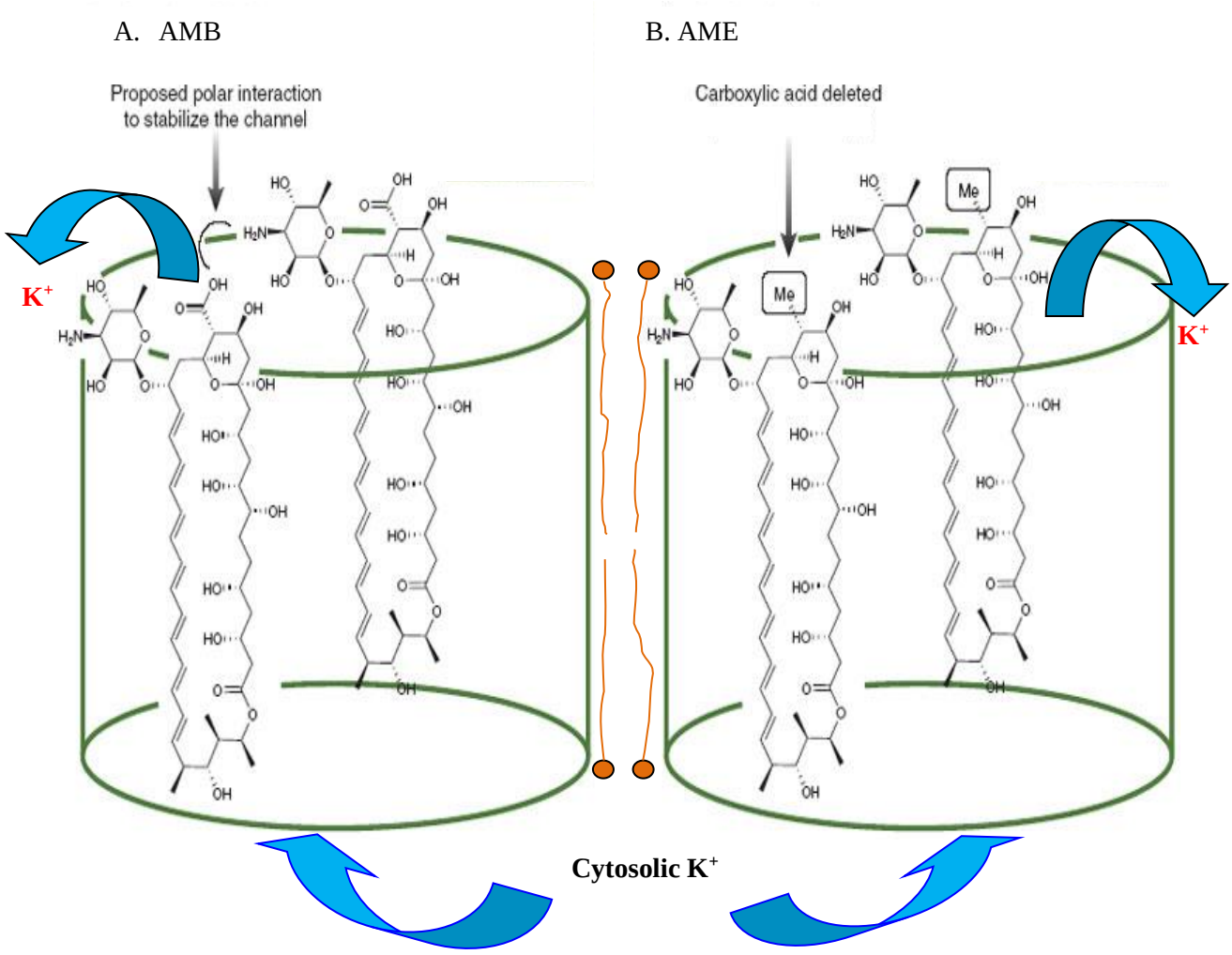
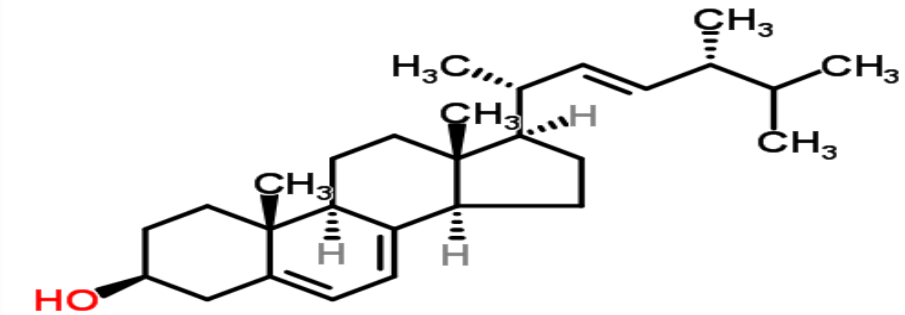
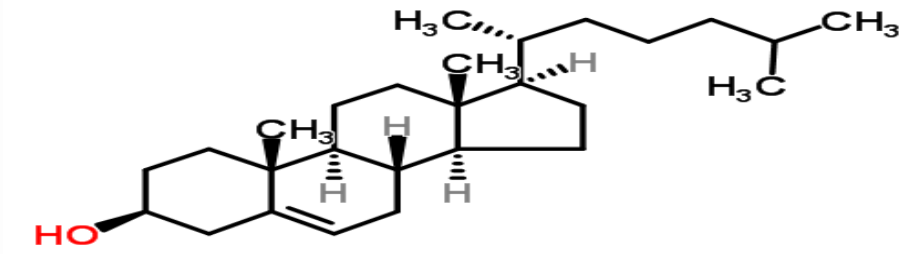


Figure 1.4 Representative structures of fungal ergosterol and mammalian cholesterol

A. ErgosteroL



B. Cholesterol



1961; Schaffner and Mechlinski, 1972). Unfortunately, parenteral AMB administration, even as Fungizone®, the commonest clinical preparation, is associated with dose-limiting nephrotoxicity (Brajtburg et al., 1996).

Parenteral AMB has a unique pharmacokinetic (PK) property, which provides clues regarding its reported human toxicity. This knowledge derives mainly from preclinical animal studies and information distilled from long-term clinical use of AMB. AMB clearly has a low therapeutic index, and the dose of AMB required to kill 50% of experimental mice (LD_{50}) when given i.v is 4.5 mg/kg (Schaffner and Mechlinski, 1972). A three-compartment physiology-based pharmacokinetic model (PB/PK) best illustrates (Figure 1.5) the distribution kinetics of AMB after a single i.v injection in disease-free non-human primates (Jagdis et al., 1977) and chronic i.v administration in humans (Atkinson and Bennet, 1978). The initial plasma elimination rate for AMB is about 24 hrs and the terminal elimination phase is approximately 15 days (Atkinson and Bennet, 1978; Lawrence et al., 1980). The long terminal $t_{1/2}$ of AMB is associated with its binding to tissue proteins (Atkinson and Bennet, 1978; Berkesky et al., 2002). This high tissue binding makes AMB linger in tissues for prolonged periods, in some cases for up to twelve months (Janknegt et al., 1992). Alterations in protein binding can alter the distribution and elimination of drugs and thus their availability and toxicity (Lewis and Wiederhold, 2003).

AMB's distribution to renal tissue is thought to contribute to its kidney toxicity (Berkesky et al., 2002). How AMB accumulates in the kidneys is poorly understood, though its moderate to high molecular weight of 924.08g suggests it may be due to a transport mechanism, evidence to support this hypothesis is lacking. Romanini et al

(1999) suggested that renal accumulation of AMB is encouraged by the formation of the toxic AMB polymers due to the acidic environment of urine in the renal tubules. How AMB gets into the tubules has not been fully elucidated, especially as its high plasma lipid/protein binding (> 90%) appears to impair its glomerular filtration, as reflected by its low renal tubular excretion of only about 3% of the unchanged drug (Lawrence et al., 1980).

Nevertheless, renal vasoconstriction following AMB's administration has been suggested as an indirect mechanism of its renal toxicity. This is understood to result from inappropriate tubulo-glomerular feedback loop induced by AMB's permeabilization of macula densa cell membranes in the distal tubule with consequent afferent arteriolar vasoconstriction and reduction in GFR (Sabra and Branch, 1990). Alternately, *in vitro* studies suggest AMB acts directly on the renal vessels themselves by altering intracellular calcium levels as evidenced by the reversal of AMB-vasoconstriction using calcium channel blockers (Sabra and Branch, 1990; Sawaya et al., 1991; Fanos and Cataldi, 2000). Furthermore, AMB-nephrotoxicity is well understood to result from its direct membrane permeability effects on tubular epithelium, subsequently leading to permanent tubular damage and renal failure. However, clinical evidence shows that in some cases the renal damage can be reversed by AMB-withdrawal.

Varlam and colleagues (2001) provide experimental support that AMB-renal damage can be reversed, demonstrating chemoprevention of a dose dependent AMB-induced apoptosis in rat kidneys and mammalian kidney tubular cell lines. This was consistent with (Odabasi et al., 2009). Similarly, Buitimea and colleagues (2007) using male Balb/c mice dosed with 4 mg/kg b.w of AMB every 3 days for 10 days,

demonstrated apoptotic change, membrane alterations and mitochondrial changes in renal tubular cells. AMB-induced apoptosis can contribute to its toxicity in mammalian cells other than the kidney (Marklund et al., 2001). In fact, studies in *Candida* species suggest that AMB can cause apoptosis in other eukaryotes like fungi (Phillips et al., 2003; Al-dhaheri and Douglas, 2010). Likewise, AMB-apoptosis has been demonstrated in *Lieshmenia* species, a unicellular protozoan that causes leishmeniasis (Moreira et al., 2011). Taken together, it is safe to conjecture that apoptosis may contribute to AMB toxicity, particularly in the kidneys.

The observation by Lawrence and coworkers (1980) of an insignificant accumulation of radiolabeled AMB in the brain and cerebrospinal fluid (CSF) after a single i.v injection was expected. This poor CNS penetration of about 0.03-0.3% of total dose of drug given (Collette et al., 1989) of AMB does not change over time (Monji et al., 1975). However, AMB levels up to 30% of total dose given has been detected in brain tissue of rabbits (Groll et al., 2000). The fact that the brain is a privileged site surrounded by a selectively permeable membrane, the blood brain-barrier (BBB), makes it potentially difficult for AMB to penetrate into the CNS. The intact BBB presents two epithelial barriers that AMB has to traverse in order to get to the cerebrospinal fluid or the brain parenchyma; one is the choroid plexus and the other, the cerebral vascular endothelium (Kethireddy and Andes, 2007). Both epithelial barriers characteristically have tight junctions that measure below 20 Å (vs. 100Å in other tissues), a size that effectively prevents large molecular weight compounds such as AMB (924.08 g/m) from diffusing into the brain. AMB's binding to serum proteins, which have a large size, further precludes its entrance into the brain.

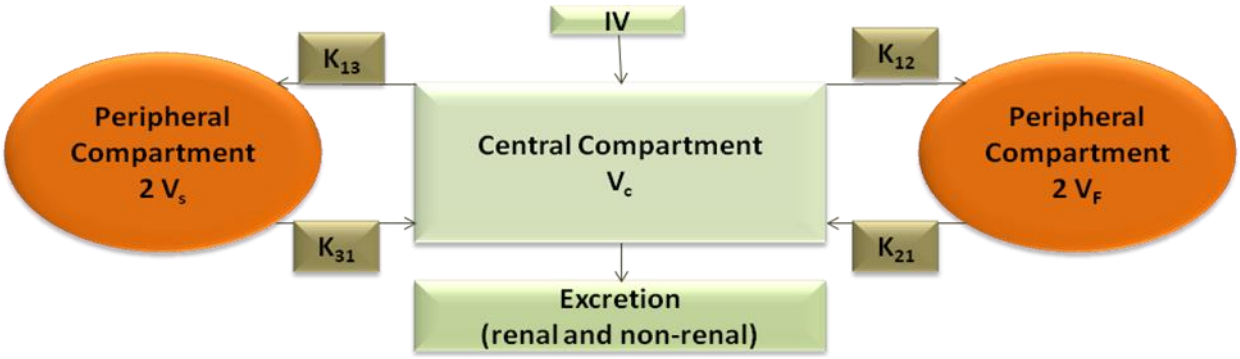
It is possible that AMB may diffuse into CNS when the BBB becomes patent. CNS inflammation due to infections compromise the BBB allowing large molecules such as albumin diffuse into the brain and CSF. The fact that AMB is highly bound to plasma proteins such as albumin may help explain how AMB can access the brain in disease states such as meningitis. Diffusion of molecules into the brain is most efficient below the upper limit of 300-400 Da when the BBB is intact (Kethireddy and Andes, 2007), however, this will likely vary in infections of the brain. Efflux pumps (P-glycoproteins) exist in the choroid plexus to limit accumulation of large MW drugs in the brain, however, AMB is not a substrate of these transporters (Kethireddy and Andes, 2007).

Early clinical use of AMB was associated with diverse neurological dysfunction, manifesting variously as headaches, visual and hearing impairment, seizures, confusion, toxic delirium, psychosis, depression, dysphoria, drowsiness and coma to mention a few (Winn, Bower and Richards, 1979; Schaffner, 1984; Hoeprich et al 1985). In addition, Peripheral myelin injury was reported with the use of AMB (Haber and Joseph, 1962).

How AMB is removed from the brain is unclear, just as there is a paucity of data on the metabolic pathway for the degradation of AMB in the body (Lemke et al., 2005). Bekersky et al (2001) using liquid chromatography tandem mass spectrometry technique suggested that metabolism played only a minor role in AMB excretion in humans. Using mass balance calculations they were able to account for over 90% of unchanged AMB, excreted mainly (70%) in the feces (biliary excretion) with 20% excreted in urine at 1 week in patients treated with AMB. In addition, no potential metabolites were detected in the urine or feces.

Figure 1.5 Pharmacokinetics of AMB

Three compartment open pharmacokinetic model of AME/AMB. This is modeled in Rhesus monkey. The rate constants are represented by subscripts of K. (Modified from Jagdis et al., 1977)



- Species- Rhesus monkey
- K_{10}; AME/AMB, 0.1221/0.0290 (Ratio, 4.21)
- VF-Fast compartment
- $K_{12}K_{21}$; AME/AMB, 0.3493, 0.2780/ 0.7932, 0.5715
- VS-Slow compartment
- $K_{13}K_{31}$ AME/AMB, 0.0162, 0.0613/ 0.0108, 0.0612
- Ratio of mean peak plasma level (30 min) after i.v AME/AMB (5mg/kg; 1mg/kg)

  =14.98/1.3 µg/ml = **11.98**

1.1.4. Clinical Applications of AME in Coccidioidomycosis

1.1.4.1 Discovery and Introduction of AME

Vigorous efforts to overcome the serious human safety concerns of AMB yielded potential success in the discovery/invention of its derivative AME and its water-soluble salts (Mechlinski and Schaffner 1972). AME generated interest amongst researchers and clinicians at the time because it retained the full antifungal properties of its parent AMB (Mechlinski and Schaffner, 1972; Schaffner C.P., personal communication). Most of all, interest in AME grew because it was demonstrably less toxic than AMB and specifically lacked appreciable kidney toxicity.

Why is AME less toxic than its parent drug AMB? Like AMB, the answer may be derived from physical, pharmacologic, and pharmacokinetic properties of AME. Comparing the properties of both drugs can derive additional toxicological clues, because AMB is closely related to AME (Figure 1.2) and has been more extensively studied. It is evident from *in vitro* and *in vivo* studies that AME and AMB share a similar mode of action (Figure 1.3) on eukaryotic membranes (Schaffner and Mechlinski, 1972). Cass and coauthors (1970) demonstrated in cholesterol-membrane experiments, that AMB and AME share a comparable propensity for permeabilizing membranes. As expected, eukaryotic membrane binding of both drugs, like that of other polyenes, is modified by cholesterol, such as seen when fetal bovine serum is added to growth media of cells incubated with the drugs (Bonner et al., 1976).

Chemical studies demonstrated that AME's aqueous solubility confers a lower toxicity than AMB in experimental animals (Keim et al., 1976), including non-human primates (Jagdis et al., 1977), as well as diverse cell culture models (Racis et al., 1990). According to Schaffner and Mechlinski (1972), the methyl ester salts, unlike AMB, possess excellent aqueous solubility (75 mg/ml) as measured by spectrophotometer. The esterification of the free carboxylic acid of AMB to form AME disrupts the zwiterrionic characteristics of AMB. Thus, AME, unlike AMB, has a net charge at normal body pH of 7.4 (Mechlinski and Schaffner, 1972). Despite its excellent solubility, AME cross membranes with varying degrees of difficulty.

Comparative pharmacokinetic studies provide further clues as to why the polyenes AME and AMB differ in their toxicity. Intravenously injected AME's decline in serum is represented by a three-compartment PB/PK similar to AMB (Figure 1.5). Contrasting AMB, AME has a shorter biphasic half-life of 2.5 hours and biological half-life of 4 days (Jagdis et al., 1977; Lawrence et al., 1980). Furthermore, much like AMB, distribution of AME to the brain is limited, on average 1.7 % of total AME given i.p is detected in brain over 96 hrs (Monji et al., 1975). Bioautographic and thin layer chromatography analysis (TLC) of radiolabeled AME (C^{14}-AME) of non-diseased mice demonstrate that AME is not de-esterified or metabolized in the body (Monji et al., 1975; Lawrence et al., 1980). In addition, Monji and colleagues (1975) demonstrated that elimination of AME from the body is mainly in urine as the unchanged drug.

The lower clinical acute toxicity of AME compared to AMB has been reported. Experimental investigations in mice, rats and dogs support the fact that AME, with notably reduced kidney toxicity, is considerably less toxic than AMB (Bonner et al.,

1972; 1975; Keim et al., 1973; 1976; Lawrence and Hoeprich, 1976; Gadebusch et al., 1976; Schaffner C. P., personal communication). The dose required to kill 50% of experimental mice (LD_{50}) for AMB when given i.v is 4.5 mg/kg compared to 75 mg/kg b.w for AME (Schaffner and Mechlinski, 1972). These LD_{50} values are comparable to those in a related study in mice, implying that AMB (6 mg/kg) has a higher likelihood of causing toxicity than AME (85 mg/kg) when given i.v (Lawrence and Hoeprich, 1976). In addition, when given i.p in mice, AME at 400 mg/kg led to death in only 50% of the animals evaluated, compared to 99 mg/kg of AMB that induced 100% lethality (Schaffner and Mechlinski, 1972). Non-human primate studies (Lawrence et al., 1979; Jagdis et al., 1977) further support the premise that AME is significantly less toxic than AMB.

Several cell culture experiments lend further support that AME is less toxic than AMB. For example Fischer and coworkers (1975) demonstrated a significantly lower toxicity for AME, evidenced by increased 24-hr survival, increased 72-hr viability and increased growth rates in six different non-neural cell lines derived from normal and tumor cells of human and murine hosts. In another study, Fisher et al (1976) further demonstrated the reduced *in vitro* toxicity of AME by measuring 24-hr survival, 72-hr viability, growth rate and early membrane permeability using ^{51}Chromium release in five different cell lines. They observed a greater toxicity with AMB compared to AME in the five different cell lines drawn from turtle, marsupial, human, rabbit and hamster, implying AME may have a better safety profile. Indeed, Bonner et al (1976) found AMB more cytotoxic than AME after comparing both drugs *in vitro* with various concentrations of serum using membrane permeability (^{51}chromium release) as a measure

of toxicity. In support, Racis et al (1990) demonstrated up to a ten-fold lower toxicity for AME than AMB in several non-neural human cells in culture, consistent with the conclusions that AME is much less toxic than AMB.

Further *in vitro* studies provide evidence that cytotoxic effects in fungi for AME and AMB may be similar. Huston and Hoeprich (1977) compared the biological activity of AME and AMB on *Coccidioidomycosis immitis* using *in vitro* models and found no statistical difference for drug needed to kill 50% of the colony of the pathogens or the minimal inhibitory concentrations (MIC). Their observed MIC for both compounds (AMB 0.99 µg/ml versus AME 0.88 µg/ml) was consistent with AME's comparable efficacy to AMB (Mechlinski and Schaffner, 1972; Bonner et al., 1972; 1975; 1975; Howart et al., 1975; Gadebusch et al., 1976; Lawrence and Hoeprich, 1976; Huston and Hoeprich, 1978; Oblack et al., 1981).

1.1.4.2 The Hoeprich Clinical Study of AME

Following the confirmation of the antifungal efficacy of AME and its significantly reduced toxicity at the Squibb Institute, the Waksman Institute provided Squibb authorization to produce commercial quantities of clinical grade AME (Schaffner C. P., personal communication). Researchers at the University of California-Davis subsequently pursued its clinical assessment in humans. With subsequent FDA approval, full improvement was recorded with a patient treated with AME at the University of California. Interestingly, this pilot case of AME chemotherapy was terminally ill with

disseminated Coccidioidomycosis (Valley Fever) and had failed to respond to AMB and several other non-investigational drugs. Subsequent AME successes with other seriously ill patients amid minimal side effects generated great interest (Schaffner C. P., personal communication).

AME's early clinical success led to use in a larger clinical evaluation, with FDA approval based on compassionate grounds (Schaffner C. P., personal communication). In 1975, AME was used to treat a cohort of fifty-three patients at the University of California-Davis Hospital who contracted life-threatening disseminated 'valley fever', including meningo-cerebral involvement, after a dust storm in California (Schaffner C. P., personal communication). Although the increased incidence of fungal disease amongst this cohort was caused by eight different fungal genera (Hoeprich et al., 1988), over 70% of the patients were infected with only *Coccidioides*, particularly the highly lethal meningo-cerebral type.

As noted in section 1.1.4.1, AME is highly effective against Coccidioides. Based on its efficacy, fifty out of the 53 infected cases were recruited into the AME protocol. Enrollment was based on three criteria: 1) failure of non-investigational drugs, wherein the patient failed to improve and/or relapsed on cessation of therapy due to adverse reactions; 2) development of severe renal dysfunction with existing therapies 3) pulmonary involvement as the primary localization of the fungal infection. AME was used in the trial at empirical doses up to five times that necessary to attain fungal kill. About 70% of the patients who met the recruitment criteria into the AME protocol benefited from the AME treatment (Table 1.1). This is noteworthy considering that most of the patients recruited were deemed terminally ill and many were already suffering

from the debilitating toxicities of the first line drugs, particularly AMB (Hoeprich and coworkers (1988). These observations support prior *in vitro* and experimental animal efficacy studies that AME is a potent broad-spectrum antifungal agent.

1.1.4.3 Controversy over Human Use and Blacklisting of AME

Unfortunately, fourteen cases from the AME trial came to autopsy. Histopathologic analysis of brain sections by Ellis and coworkers (1982) revealed diffuse leukoencephalopathy in 50% of the cases. The leukoencephalopathy was attributed to the AME. Following these reports, FDA terminated use of AME, even on a compassionate IND basis. Subsequent analysis revealed limitations to the Ellis et al. report, such as the fact that there were no comparative AMB groups in the trial, in addition to its 'unblinded' design (Hoeprich et al., 1982) and a possible overestimation of the incidence of diffuse leukoencephalopathy that led to the AME embargo. In support of a possible overestimation, a blinded parallel histological analysis of comparable sections from the AME trial performed by the Armed Forces Institute of Pathologist (AFIP) contended with Ellis and coworkers (Hoeprich et al., 1988). Nevertheless, the working interpretation that AME induces leukoencephalopathy had become entrenched in clinical literature.

Table 1. 1 Outcome of AME treatments in the Hoeprich clinical trial (Hoeprich et al., 1988)

	Outcome of AME Rx	**Number**	**%**
1	Cure	16	32
2	Persistent improvement	9	18
3	Temporary improvement	9	18
4	Death (mycotic)	14	28
5	Death (non-mycotic)	2	4
	Total	50	100

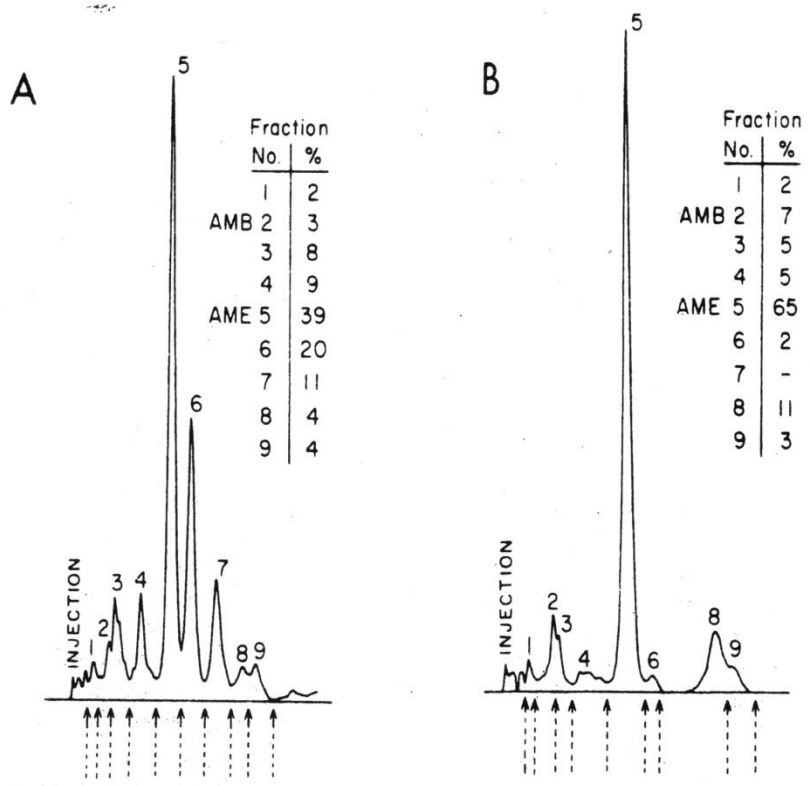
Cure: defined as absence of fungal activity after 5 years of treatment cessation (assessed by clinical and laboratory surveillance).

Persistent Improvement: defined as absence of fungal activity for up to 6 months while on treatment (assessed by clinical and laboratory surveillance).

Temporary improvement: defined as reoccurrence of fungal activity after 6 months of treatment (assessed by clinical and laboratory surveillance).

Figure 1.6 Representative Chromatograms of Impure AME Lots Used in Human Trials

Nine batches of AME preparations (Squibb Institute of Medical Research, Princeton, N.J) were used therapeutically in human trials and experimental animal studies where AME was associated with neurotoxicity. (A) Lot # NN005NA is representative of the first six batches (NN009NB; FGN800H/A; NN010NB; NN005NA; NN006NA; NN007NA). (B) Lot # NN011ND, representative of the last three batches used and includes NN009ND and NN010ND (Hoeprich et al., 1980). The method of synthesis was changed for these batches, leading to a variation in content of some of the multi-methylated derivatives (absence of component 7 and reduction of component 4 and 6) with an increased yield of AME.



1.2 Re-evaluation of AME for Clinical Use

1.2.1 Contamination as Cause of Prior AME-induced Neurotoxicity

Indeed, the widely held perception in clinical literature that AME is neurotoxic was upheld despite subsequent discovery of contamination in the AME preparations used in the trials. Suspicion of inconsistencies in the lots supplied (Hoeprich et al., 1988) led to the examination by high-pressure liquid chromatography (HPLC) at Rutgers University, revealing contamination of the AME lots used, with AMB (Figure 1.6), often in high concentrations (up to 8%), including several multi-methylated derivatives of AMB (Hoeprich et al., 1982; 1988). Further analysis (Rinehart, 1982) confirmed these HPLC findings, casting little doubt that all AME lots used in its trials were heavily contaminated.

Half of the 14 autopsied cases with evidence of leukoencephalopathy had received AMB and undetermined amounts of the multi-methylated derivatives of AMB by the end of the trial. Table 1.2 depicts the source and quantification of AMB amounts received by the 14-cases. It is unclear what role the cumulative AMB, or the other AMB-derivatives as contaminants of AME may have played in the complex toxic responses observed in the AME-treated patients. According to Hoeprich and coworkers (1988), several patients had developed side effects that included hemolysis, ototoxicity and neuropsychiatric symptoms that were similar to but milder than that seen with AMB use. In addition, neurotoxicity manifesting as diffuse leukoencephalopathy was evident in the

Table 1. 2 The summaries of CNS pathology, cumulative AME doses given and corresponding AMB doses given as contaminants of the AME preparations used in the Hoeprich-trials of AME. All cases of significant leukoencephalopathy appear to have received at least a cumulative dose of 10g of low purity AME. 89% of all cases with leukoencephalopathy had received treatment using at least 2 different lots of AME. Each AME lot contained different percentages of AMB and its multi-methylated derivatives.

Autopsy Case	Leukoencephalopathy	Total AME Dose given (mg)	AMB content of AME (mg)	Number of different AME lot received
1	**Yes**	19,266	541	3
2	**Yes**	10,482	467	3
3	**Yes**	14,000	680	2
4	**Yes**	12,013	961	2
5	**Yes**	9,846	282	3
6	**Yes**	11,018	253	3
7	**Yes**	22,050	815	3
8	**No**	7,200	576	1
9	**Mild**	5,950	357	1
10	**Mild**	4990	131	2
11	**No**	1400	112	1
12	**No**	875	87	1
13	**No**	906	45	1
14	**No**	NA	NA	1

post mortem cases that had received cumulative doses of impure AME in excess of 10g (Table 1.2).

Significant neurotoxicity has been associated with tainted AME (Ellis et al., 1982; 1988) and with AMB (Liu et al., 1995). This raises three important questions: first, was AME responsible for the observed diffuse leukoencephalopathy? Second, could the leukoencephalopathy have been due to AMB? Finally, was the leukoencephalopathy a result of drug interactions between AME and AMB or the other contaminants? These critical questions have remained the subject of debate in literature 30 years later.

The fact that AMB can induce CNS toxicity adds to the controversy generated over the potential neurotoxicity of AME and the role of each drug in the etiology of neurotoxicity in AME's trial. The ambiguity of the relative neurotoxicities of AME and AMB has prompted investigational neurotoxicity studies to evaluate the two polyenes.

1.2.2 In Vivo Investigational Neurotoxicity Studies

Hoeprich, Huston and Wolfe (1985) using disease-free mongrel dogs, compared the neuropathology of sub-chronically administered AMB with that of AME preparations similar to form used in the human trials of AME. Clinical doses of AMB at 1.25 mg/kg b.w led to a 100 % death, warranting a dose reduction. The reduced AMB dose of 0.75 mg/kg was associated with a 40% survival, severe weight loss and notable

nephrotoxicity. Myelin injury was also observed in 60% of the surviving AMB-brains, although this was focal in nature (vs. diffuse in AME human trials). The AME-dogs received 10 mg/kg b.w, a dose 2 times higher than that used in its human trials. Although the dogs tolerated the high AME dosing without significant signs of weight loss or nephrotoxicity, about 40% of them had histopathological evidence of diffuse leukoencephalopathy, consistent with the findings in the AME human trial. Some of the control dogs (33%) developed myelin lesions similar to the neurotoxic form of AME, suggesting the need for further neurotoxicity evaluations of both polyenes.

In another canine study, Ellis and coworkers (1988) using disease free dogs (2 German short haired pointers and a pit bull) assessed neuropathological findings following administration of AME (57.2% AME content) that was consistent in purity with the preparations used in the human trials. Employing an AME dose (between 299-300 mg/kg b.w) sixty times the usual clinical dose of 5mg/kg b.w, they demonstrated clinical signs and histopathologic evidence of diffuse leukoencephalopathy in all the AME dogs that was consistent with the AME human trials. The neurotoxicity was defined as oligodendrocyte depletion, accumulation of lipid-filled macrophages, fibrillary astrogliosis and swelling or fragmented axons, noticeable in sub cortical white matter of frontal lobes and the centrum ovale. The preceding canine studies suggest that low purity AME preparations that are contaminated with AMB and multi-methylated AMB derivatives can be neurotoxic.

In contrast to the preceding canine studies, Reuhl et al (1993) using highly purified AME preparations (>93% AME with <1.3% residual AMB) demonstrated that AMB, but not AME is neurotoxic. Five days following the intraventricular injection of

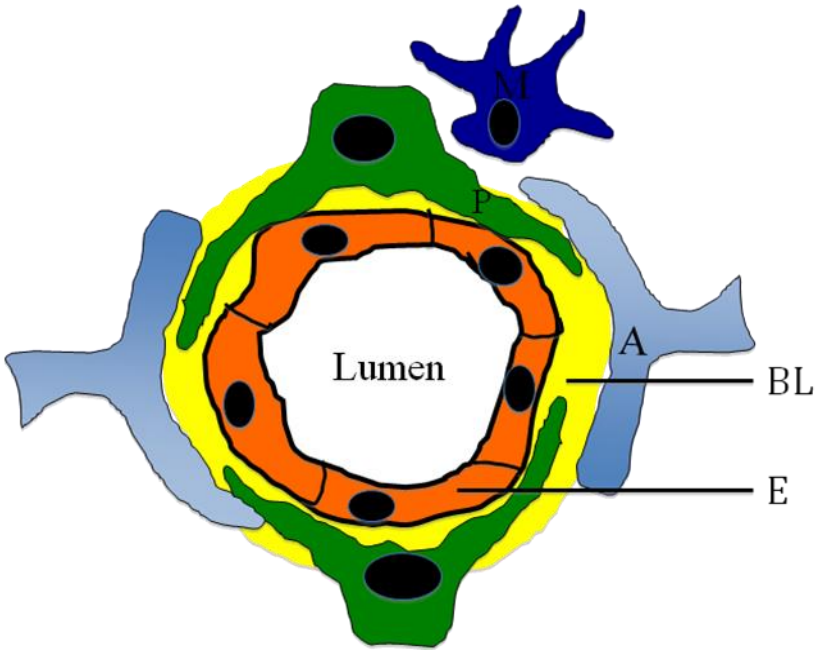
single doses of AMB (0.01, 0.1, 1.0, 2.5 and 5 mg/ml) and AME (0.01, 0.1, 1.0, 2.5, 5.0 and 50 mg/ml) into the CSF of adult female Wistar rats, they observed myelin damage at 1 mg/ml AMB using light and electron microscopic assessment. The severity of the myelin damage, based on ultrastructural changes were observed as intra- and intermyelinic edema, loosening of myelin lamina and separation of fascicles was dose-dependent and was associated with neuronal and astrocytic swelling that included chromatin clumping. This suggested the outcome was not distinct to one cell type. In addition, 33 % of the rats given the highest dose of AMB (5 mg/ml) died, with the surviving animals suffering from severe weight loss (18%) and lethargy. In Contrast, the highest concentration of AME used (50 mg/ml) caused no deaths with a less severe weight loss (14 %) and lethargy. Furthermore, the lowest AME dose to cause myelin injury was 5 mg/ml. The myelin and adjacent cellular injury was similar to 5 mg/ml AMB, although less severe.

Reuhl and coworkers (1993) also compared the neurotoxicity of daily intravenous injections of AMB (5 mg/kg b.w) and highly purified AME (50 mg/kg b.w) over a 5-day period. Light and electron microscopic assessment of these sub-toxic doses of AMB and AME (LD_{50}; 6 mg/kg b.w vs. 86 mg/kg b.w respectively) revealed myelin edema in the cortical white matter of AMB-treated rats following 5 days cessation of treatment. In addition to white matter edema, neuronal injury was apparent with edema of adjacent glial cells. Although the CNS endothelial cells appeared normal, the increased lysosomal content and swelling of white matter perivascular pericytes and edema of perivascular astrocytes suggests AMB may directly compromise the BBB. Both perivascular

astrocytes (covers 90% of brain endothelium) and pericytes (covers 80% of brain endothelium) are vital to the maintenance of the BBB integrity (Figure 1.7).

Figure 1.7 Representative diagrams of the components of the BBB

The BBB or neurovascular unit is represented by; (A) Astrocyte end feet; (B) Basement membrane; (E) vascular endothelium; (P) pericytes, (M) microglia; (N) neuron. The endothelial cells are specialized cells with tight junctions that prevent easy diffusion of drugs into the brain. (Modified from ElAli et al., 2014)



AMB relative to AME was also toxic to peripheral nervous system myelin (Reuhl et al., 1993). This was consistent with Gold and coworkers (1989; 1990). Employing transmission electron microscopy, these studies provided experimental evidence of neurotoxicity due to AMB, demonstrated by nerve injury associated with demyelination in the PNS.

Additional data from Reuhl et al 20 years ago demonstrates that AMB as a contaminant of AME potentially induces leukoencephalopathy. Neurotoxicity was recorded after injecting adult female Wistar rats with daily weight for weight i.v combination of AMB-AME (1:9) at 50-mg/kg b.w for five successive days. Intravenous AME at this dose is relatively nontoxic as discussed in the preceding paragraph, but the mixture resulted in signs of neurotoxicity in five out of six rats (86 %). The myelin lesions were less severe than seen in the group treated with AMB only. These results signify that AMB as a contaminant of AMB increases the chances of AME causing leukoencephalopathy, and that the severity of the white matter injury may vary according to the extent of AMB contamination.

The cumulative animal investigation results indicate a need for more studies that critically evaluate the issue of relative neurotoxicity of the polyenes. Unfortunately, economic and logistic issue besets research into new antibiotics development. Since the Reuhl et al. study, further studies evaluating the neurotoxicity of clinically relevant doses of AME have been lacking. Prior canine studies visibly ‘overdosed’ their animals with AME. A rigorously controlled canine study along with more laboratory animal

investigations would provide valuable insight to the contentious issue of polyene neurotoxicity. Further benefit would be derived from studying neurotoxicity of pure AME or AMB in non-human primates, a model phylogenetically closest to humans. Supplementary data from an unpublished observation in rhesus monkeys by Hoeprich et al., further demonstrated the absence of leukoencephalopathy after treatment with AME (ctd. in Hoeprich, 1982).

1.2.3 *In Vitro* Neurotoxicity Studies

Racis et al (1990) were first and perhaps the only authors to compare the two antibiotics *in vitro,* demonstrating that AMB is more neurotoxic than AME. They exposed AMB and pure AME (< 0.1 percentage AMB; > 90 percentage AME) to primary culture of rat cortical cells enriched with oligodendrocytes and astrocytes. They confirmed a differential cytotoxicity of both drugs by employing immunofluorescent techniques. They concluded that AMB demonstrated dose-dependent oligodendrocyte toxicity, through membrane pores effects. The membrane perturbation in oligodendrocytes was sensitive to low concentrations of AMB (0.1µg/ml) and appeared to be irreversible, leading to cell death. In sharp contrast, AME induced insignificant oligodendrocyte cytotoxicity at concentrations higher than 10 µg/ml. This difference in membrane-induced cytotoxicity may indicate a vulnerability of the sterol-rich oligodendroglial cells to sterol-targeting polyenes, which is greater for AMB than highly purified AME.

Table 1. 3 Summary of studies that investigated the neurotoxicity of AME and AMB

Study	Experimental Model	Drug	AME Purity	AMB content of AME	Route of Administration	Neuro toxicity	Myelin /oligodendrocyte injury	Author
1a.	Rat	AMB	NA	NA	i.t	Yes	Myelin	Reuhl et al., 1993
B.	Rat	AME	93%	1.3%	i.t	No	-	Reuhl et al., 1993
c.	Rat	AMB: AME (10%:90 %)	93%	1.3%	i.v	Yes	myelin	Reuhl et al., 1993
2a	Dog	AMB	NA	NA	i.p	No	-	Ellis et al., 1988
b.	Dog	AME	67%	8%	i.p	Yes	Oligodendrocyte/myelin	Ellis et al., 1988
3a.	Monkey	AMB	NA	NA	i.v	No	-	Hoeprich et al., 1982
b.	Monkey	AME	NA	NA	i.v	No	-	ctd. in Hoeprich, 1982
4a	Primary mixed neuronal cell culture	AMB	NA	NA	NA	Yes	Oligodendrocyte/myelin	Racis et al., 1990
4b.	Primary mixed neuronal cell culture	AME	>90%	<0.1%	NA	No	-	Racis et al., 1990

Racis et al (1990) also observed a greater *in vitro* toxicity of AMB towards sterol rich myelin than AME. They studied the direct action of both drugs on myelin sheet formation and maintenance in culture and showed that AMB disrupted already compacted myelin and prevented myelin sheath formation at concentrations that were ten-fold higher than for AME. These *in vitro* data adds to the accumulating evidence in animals (Reuhl et al., 1993) and humans (Walker and Rosemblum, 1992; Liu et al., 1995) that AMB is more neurotoxic than AME

1.3 Statement of Hypothesis and Significance

Demyelination is the prominent feature associated with AMB and AME neurotoxicity, manifested as leukoencephalopathy (Hoeprich 1985; Racis et al., 1990; Reuhl et al., 1993). We have chosen to focus both on myelin and on the myelin forming oligodendrocytes in this thesis. We believe the peculiar metabolic needs of oligodendrocytes and the high membrane cholesterol content of myelin contribute critically to their vulnerability to toxicants in the CNS, leading to their central role in the pathology of demyelination.

Based on studies showing that AME preparations associated with leukoencephalopathy were extensively contaminated by amphotericin B, along with recent evidence that AMB causes significant myelin toxicity and oligodendrocyte injury, **we hypothesize that amphotericin B is damaging or lethal to myelin and oligodendrocytes by inducing plasma- and mitochondrial membrane-permeability.**

Furthermore, pure amphotericin methyl ester (AME) at clinical concentrations will not be toxic to myelin or oligodendrocytes.

Specifically, the hypothesis will be tested by evaluating the following questions:

1) Does *in vitro* exposure to varying concentrations of AMB and AME lead to plasma membrane or mitochondrial membrane permeability?

(a) Is there a difference between AMB and AME induced membrane permeability in differentiated human MO3.13 oligodendroglia?

(b) Is the loss of differentiated human MO3.13 oligodendroglia plasma membrane permeability by AMB and/or AME, related to nuclear morphological changes consistent with apoptosis?

(c) Is the loss of mitochondrial membrane potential an event known to result from mitochondrial membrane permeability related to AMB or AME exposure and is there concomitant release of cytochrome *c*?

(d) Can the cells be rescued from injury by chemoprevention of mitochondrial membrane potential loss?

This project aims to unravel the differential sensitivity of mature oligodendrocyte *in vitro* to AMB and AME, and to define possible molecular mechanisms by which AMB or AME may affect normal oligodendrocyte viability and thus myelin sheath maintenance. Ultimately, these studies may provide insight into the potential impact of the macrolide antifungal antibiotics on normal tissues of the CNS. Furthermore, data from these studies can be used as a guide to re-characterize the safety of AME (free of impurities).

The first aim of this thesis (Section 1.4) is to carry out a preliminary study in experimental mice to determine the feasibility of identifying the neurotoxic potential of AME or AMB in our subsequent *in vitro* study. In addition the study will help evaluate efficient methods to be used in cell culture study. The second aim (Chapter 2) is to evaluate the *in vitro* effects of pure AME compared to AMB in an immortalized cell-line, with an emphasis on the potential perturbation of the lipid-rich plasma membranes of oligodendrocytes as a basis of possible toxicity. Finally, Chapter 3 compares AMB and AME putative effect on the oligodendrocyte mitochondrial-membrane permeability *in vitro*.

1.4 Preliminary Data on The Comparative Neurotoxicity of AME and AMB in Mice

1.4.1 Abstract

The purpose of this *in vivo* study was to assess the feasibility of carrying out a mechanistic study of Amphotericin B methyl ester (AME) and Amphotericin B (AMB) potential to induce neurotoxicity. An intraperitoneal administration of a 5 mg/kg of body weight of AMB (sub-lethal dose) and a similar dose of AME (reflects its clinical dose but i.p vs. i.v) that was highly purified (93 % AME and < 1.3% AMB) in adult female Balb/c mice every two days for 28 days demonstrated AMB to be more toxic than AME. AMB-mice demonstrated a higher weight loss than AME. Light and fluorescent microscopic analysis showed evidence of receding myelin, axonal stripping in AMB-treated mice. Decreased fluoromyelin fluorescence in the cerebral white matter indicated myelin loss,

seen more in AMB-brains than AME. Similarly treated AMB- and AME-mice, injected for two months, followed by 3 months of drug-free recovery, provided additional evidence that AMB induced myelin toxicity but AME did not. Nuclear morphological analysis of brain sections revealed nuclear chromatin condensation, margination, and fragmentation reflective of apoptotic change in AMB-brain sections after a month and following 2 months exposure followed by 3 months of cessation of treatment. The computed nuclear area factor (NAF), a quantitative fluorescent measure of cell apoptosis, indicated AMB-induced apoptotic change. No similar observation was noted in AME-treated mice. Light and electron microscopic analysis of adult male Balb/c mice administered AME (7 mg/kg of body weight) alone for over 5 months, revealed minimal myelin injury (inter- and intramyelinic edema, myelin sheath stripping and myelin sheath disintegration) at levels similar to the 5% glucose controls. Our data suggests that clinically relevant doses of AME do not induce significant central myelin injury, but AMB induces myelin injury. Our preliminary data also reveals that AMB induces apoptosis in non-neural cells in cerebral white matter of mice.

1.4.2 Introduction

Treatment of severe systemic fungal infections is currently a significant clinical challenge. Amphotericin B (AMB), a polyene macrolide with dose-limiting nephrotoxicity very important in managing severe forms of these complex fungal disease and is often considered the “Gold Standard” of antifungal therapy (Keim et al., 1973; 1976). Amphotericin B methyl ester (AME) was developed by chemical modification of

AMB (Mechlinski and Schaffner, 1972) in an effort to overcome the many side effects of AMB. The early use of AME in its human-trials recorded significant success with hard-to-treat fungi such as *Coccidioidomycosis immitis* the etiological agent for "Valley Fever", but concerns over purity and neurotoxicity led to a halt of its use. Commonly used drugs for severe fungal disease such as AMB are very toxic or inadequate to prevent the significant morbidity and mortality that follows the disease (Mathisen et al., 2010). Recently, a nation-wide outbreak of systemic fungal infections from contaminated spinal injections led to meningitis in over 30% of infected cases, with 8 % ending in fatalities (CDC, 2014). The failure of the azoles and AMB to mitigate the reported mortality underscores the challenges managing systemic fungal infections (Mathisen et al., 2010) and the urgent need for less toxic but highly effective alternatives.

AME has a wide therapeutic index with low parenteral and *in vitro* toxicity relative to AMB. *In vitro* (Mechlinski and Schaffner, 1972; Bonner et al., 1972; 1975; Howarth et al., 1975) and *in vivo* efficacy studies (Bonner et al., 1972; 1975) support these conclusions. AME has reduced *in vitro* (Fischer et al., 1975) and *in vivo* toxicity (Mechlinsky and Schaffner, 1972; Bonner et al., 1972) justifying the initial human use of AME as an alternative to the highly toxic AMB. However, in the first and only clinical trial of AME, histopathological analysis of several cases that died of their disseminated fungal infections were found to have leukoencephalopathy (Hoeprich et al., 1988).

The reported leukoencephalopathy was primarily seen in the cerebral white matter after parenteral AME injections, bringing AME's safety into question. The lesions manifested as diffuse demyelination, with gliosis that included astrocytic proliferation (Ellis et al., 1982). The exact incidence of the diffuse white matter lesions remains

unclear (Ellis et al., 1982; Hoeprich, 1982), prompting the re-examination of the AME lots used and subsequently confirming their low-purity (Reinhart, 1982; Hoeprich et al., 1988). A significant amount of AMB (2-8%) as a contaminant was discovered in all the lots, along with numerous multi-methylated derivatives of AMB (Hoeprich et al., 1988).

In subsequent assessments of the AME trials, the issue of low-purity and contamination with AMB was largely ignored. In the first study, Hoeprich et al (1985) administered 14 mongrel dogs with either low dose i.v AMB (0.75 mg/kg b.w) and high dose AME (10 mg/kg b.w) preparations containing about 7% residual AMB for 30 days, demonstrating astrogliosis in the AME-dogs. In the second canine study, Ellis et al (1988) reported leukoencephalopathy in AME-treated dogs following 30 alternate day i.v doses. The AME preparations, taken from similar lots to those in the AME-trials were of low purity (57 % AME content), containing up t0o 8% of AMB and undefined amounts of multi-methylated AMB derivatives as impurities. Unfortunately, the presence of these impurities raises questions about the validity of the conclusions.

Results from an investigational neurotoxicity study of AME in rats demonstrated that AMB was more neurotoxic than AME (Reuhl et al., 1993). Direct injection of 1 mg/ml AMB into the brain ventricles of adult female Wistar rats resulted in ultrastructural changes reflecting neurotoxicity. Myelin damage, neuronal degeneration and astrogliosis were noted after single injections of AMB were administered. Modest signs of similar lesions were only observed with AME after concentrations (50 mg/ml), which exceeded those projected for clinical use. A similar trend was observed with intravenous administration of both drugs (AMB 5 mg/kg b.w vs. AME 50 mg/Kg b.w). In addition, i.v administration of AMB appeared be toxic to perivascular pericytes, which

are important cells that help in the maintenance of the BBB integrity. The study also showed that the AMB content of AME preparations predicted the potential toxicity of AME. Furthermore, the results showed that AMB was more neurotoxic than AME after injections into sural nerves, and that demyelination after AMB injury was not easily reversed, consistent with the studies of Gold et al (1989; 1990) in sciatic nerve of rats.

Pharmacokinetic (PK) studies in humans, non-human primates and laboratory animals suggest that AMB's long biological half-life (15 days) relative to AME (4 days) may contribute significantly to its high toxic disposition (Mechlinski and Schaffner, 1972; Monji et al., 1975; Jagdis et al., 1977; Lawrence et al., 1980; Bennet, 1995).

In vitro studies, which bypass the *in vivo* homeostatic and PK considerations, demonstrated that AMB induced more neurotoxicity than AME. Using dye exclusion methods Racis, Plescia and Schaffner (1990) demonstrated that AMB had a greater cytotoxicity than AME (>90% AME and 0.1% AMB) towards primary oligodendrocytes and astrocytes in culture. Similarly, AMB caused greater inhibition of myelin growth and regeneration in culture.

Clinical data also shows that AMB can be neurotoxic (Haber and Joseph, 1962; Carnevale et al., 1980; Winn, Bower and Richards, 1979; Schaffner, 1984; Hoeprich et al., 1985). This is important because AME is derived from AMB, and AMB is a confirmed contaminant in preparations of AME from investigative studies that cite AME as a cause of leukoencephalopathy (Hoeprich et al., 1985; 1988; Ellis et al., 1988).

AMB, as with other polyenes is known to cause cells to die by necrosis. Early reports show that AMB can cause fungi and unicellular parasites to die by apoptosis.

More recently, apoptosis has been reported with AMB use *in vitro* and *in vivo* (Marklund et al., 2000; Varlam et al., 2001) and suggested to contribute to AMB nephrotoxicity. There is no precedence in literature that AME induces apoptotic changes in kidneys. Likewise, experimental evidence of AMB or AME inducing apoptosis in the brain is lacking.

In the current study, we compared the potential neurotoxic effects of highly purified AME and AMB in mice after intraperitoneal (i.p) administration. This route was chosen vs. i.v because of the small size of mice and thus, because of the narrow lumen of the tail vein, it may easily become perforated or thrombosed (AMB causes severe thrombophlebitis) with repeat injections. From our preliminary results AME appears not to be neurotoxic at clinically relevant doses. In contrast, the commonly used AMB (Fungizone®) demonstrates neurotoxic effects. Furthermore, this preliminary data suggest the feasibility of assessing potential AMB and AME induced cytotoxicity in cell culture systems using automated image cytometry techniques is achievable.

1.4.3 Materials and Methods

1.4.3.1 Animal Husbandry

Animal studies were carried out according to the Rutgers University Institutional Animal Care and Use Committee (IACUC) approved protocols. Both males and females were used. For the 1-month, and 2-month treatment, adult virgin female Balb/c mice

(Taconic Farms, Kingston, N.Y.) weighing between 17 and 20 g at the beginning of the study were used. For the 5-month treatment, adult male Balb/c mice weighing between 17 and 20 g were used. Before the start of the experiments, mice were randomly assigned to cages, and each cage represented an experimental group. Each animal was given unique number using permanent ink marking on the tail. The mice were maintained in a standard 12-h: 12-h light/dark cycle, and had free access to water and standard mouse chow (Purina Mills, Inc., St. Louis, MO). All female mice were nulliparous and non-pregnant throughout the duration of the studies and thus, had no experience of hormonal stimulation. Hormone stimulation is suggested to alter the brain size and physiology (Oatridge et al., 2002)

1.4.3.2 Polyene Antibiotics

Amphotericin Methyl Ester (AME) and Amphotericin B (AMB), was generously donated by BioSourcePharm (Spring Valley, NY**,** purity value 93.1%, Lot # 102123) via Karykion Pharmaceuticals (Princeton, NJ). AME, supplied as the dry powder (HCl salt), contained 10 mg/vial of high-purity AME, with 1.3% of residual AMB content as determined by HPLC analysis (Figure 1.1). AMB (X-GEN Pharmaceutical Inc. NY**,** Lot # 1M6AB) was purchased from Walgreens (Somerset, NJ) with a prescription as the lyophilized powder. The dry powder of AMB was supplied sterile as Fungizone®, in vials comprising 50 mg of AMB and 41 mg of sodium deoxycholate (which can significantly alter membrane fluidity), buffered with 20.2 mg sodium phosphates (mono and dibasic

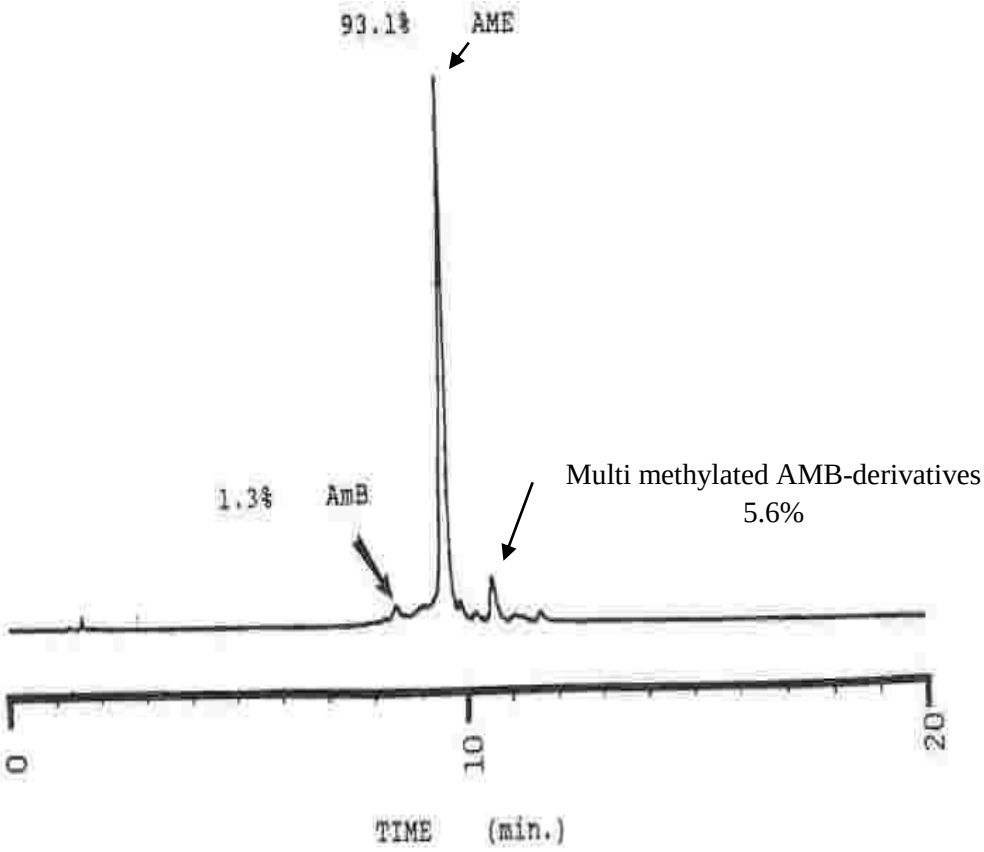
sodium phosphate, phosphoric acid, and sodium hydroxide). Both AMB and AME were reconstituted under aseptic conditions, protected from light to yield treatment solutions.

Antibiotic Reconstitution; To produce treatment solutions for comparative studies, stock concentrations of 10 mg/ml of either AME or AMB were initially prepared by suspending each antibiotic in 5% aqueous dextrose. The antibiotic stocks were then maintained in the dark at 4 ^{o}C for a maximum of two weeks, before fresh stocks were made. AME and AMB stock solutions were further diluted with 5% dextrose to give a solution of 1 mg/ml concentration prior to each injection. The vehicle (5% aqueous dextrose) served as a control.

Sterilization; All treatment solutions were purified by filtering through 0.45-µm-pore-size membranes (Lida Manufacturing Corp., Kenosha, WI). AMB forms oligomers that are < 0.45-µm in size such that use of filters smaller sized filters risk excluding potentially active AMB oligomers from the filtrate. Several investigators (Bonner et al., 1975; Lawrence and Hoeprich, 1976; Jagdis et al., 1977; Huston and Hoeprich 1978; Ellis et al., 1988; Hoeprich et al.; 1988; Reuhl et al., 1993) demonstrate that the methods of filtration, reconstitution and storage employed in this study preserve the biological activity of both AME and AMB.

Figure 1.8 Representative chromatogram of the AME Lot # 102123 used in our studies

The AMB content by High Performance Liquid Chromatography (Hewlett-Packard chromatograph model 1090 with diode detector) was less than 1.3 %. The AME preparation used, as confirmed by BioSourcePharm (Schaffner C.P, personal communication) was taken from a similar lot to that from Reuhl et al (1993). The detector was set at 408 nm.



1.4.3.3 Drug Treatment Design

AMB and AME Treatment for 1-Month To test if pure AME or AMB induces central white matter lesions (leukoencephalopathy) after 1-month, the antibiotics were administered i.p to adult virgin female Balb/c mice at a dose of 5 mg/kg of body weight (b.w) of the test animal. The i.p route was chosen for the practical purpose that it would be easier for repeat administration than the tail vein. Other researchers have also found this route appropriate (Schaffner and Mechlinski, 1972).

AMB and AME Treatment for 2-Months To test the potential progression or recovery from CNS myelin toxicity, injections of AMB or highly purified AME were given on alternate days for 2-months at a dose of 5 mg/kg of body weight.

AME Treatment for 5-Months To test the potential CNS myelin toxicity of highly purified AME after chronic administration, AME was administered i.p on alternate days to adult male Balb/c mice for 5-Months at a dose of 7.0 mg/kg b.w.

1.4.3.4 Physical Examination

To assess clinical response of mice to AME and AMB, a modified method of Irwin (1966) was employed. In brief, each mouse was directly observed undisturbed prior to injections in its group/home cage for physical appearance and viability/death. This was followed immediately by weighing, after which, animals were then placed in holding

cage. Subsequently, the desired treatment-dose to be administered was computed based on body weight of individual mice. A systematic handling and observation of each animal then followed and each was assessed for signs of neurologic (lethargy and excitation) changes. Following which, each mouse was injected with the appropriate dose of test compound. Mice were observed for a minute in the holding cage, subsequently each was returned to its original cage. All mice were observed for signs of lethargy or irritability every other day before each injection, while surveillance for death was carried out twice a day.

We measured baseline weight values (pretreatment weight) and all other weight measurements carried out were compared to the baseline values to determine changes in weight. Thus, each animal served as its own control.

1.4.3.5 Histopathology Analysis

Necropsy: Mice were euthanized after two days following the 1-month and 5-month treatment, and 3-months following the 3-month treatment, with an overdose of sodium pentobarbital i.p (100 mg/kg/body weight). All mice were perfused by transcardial infusion of 0.9% aqueous saline via the left ventricle, followed by infusion of 4 % paraformaldehyde in 0.1M phosphate buffer (pH 7.42) fixative under gravity pressure.

Tissues Processing for Histology: Brains were removed for light microscopic analysis; the left hemisphere was cut parasagittally and the right was cut coronally according to a

consensus protocol developed by the Society for Toxicological Pathology (STP) (Bolon et al., 2006). Large blocks of cerebral tissue were taken along with kidneys and post-fixed in 10% neutral buffered formalin, processed and embedded in paraplast® (Fisher Scientific). Six-micrometer thick serial sections obtained using a rotary microtome (Reichert Histo Stat 820) was mounted on ultra-frosted glass slides (Ultrastick, Goldseal; Portsmouth, NH).

Chromatic Staining: Serial paraffin sections were stained with hematoxylin and eosin using standard histological techniques (Armed Forces Institute of Pathology, 1968). To confirm myelin injury, brain sections were stained with luxol-fast blue (LFB) dye and cresyl violet using Kluver-Barrera method for myelin (Armed Forces Institute of Pathology, 1968). Light microscopic analysis was carried out using a Zeiss Axiophot microscope (Carl Zeiss GmBH, Germany).

Fluorescence Staining: Fluorescent labeling was carried out on paraffin sections of cerebral tissue mounted on slides by a modification of the method of Kanaan and coworkers (2006) for myelin and nuclear material. Briefly, serial paraffin sections were rehydrated in PBT (PBS + 0.2% Triton X-100) for 20 minutes. Subsequently the sections were soaked for 20 min in a cocktail containing three dyes. The first dye, Fluoromyelin (BrainStain™ Life/Invitrogen, Carlsbad, CA), was used to identify myelin, and the second fluorescent dye NeuroTrace™ (Life/Invitrogen, Carlsbad, CA) was used to visualize neuronal perikaryon. Both dyes were diluted 1:300 in PBS. To evaluate nuclear changes, a third dye (Hoechst 33342) was employed at a dilution of 1:500 in PBS. Sections were then washed 3x10 minutes in PBS, before cover slipping with fluoromount G (Southern Biotech, Birmingham, AL). After cover-slipping fluorescent digital

micrographs were captured using ProgRes® C14^{plus}, a cooled CCD (charged coupled device) camera (Jenoptik, GmBH, Germany) attached to a Zeiss Axiophot microscope. Images were initially captured in binary format (monochrome), and automatically pseudo-colored by the software. All images were processed with CapturePro version 2.8.8 software (Jenoptik, GmBH, Germany), and was also used to combine mirror images of sections utilizing two or three fluorescent dyes (Hoechst, fluoromyelin and NeuroTrace™ Nissl red). All images were saved electronically as an 8-bit TIFF (Tagged Image File Format) format. Subsequently, the raw TIFF images were exported to Image-Pro Plus version 7.0 for windows© software for quantitative morphological measurements.

1.4.3.6 Image Processing

Image Analysis: Cerebral sub-cortical white matter brain sections from the 1-month and 2-month treatment studies were subjected to morphometric analysis quantitative image analysis. Paraffin sections (6-µm) were obtained in series, every other section was selected for fluorescent labeling as described previously, followed by fluorescence microscopic analysis. Six sections were analyzed per brain, and for each section, six non-overlapping microscopic fields were randomly chosen for automated image acquisition. To measure number of cells/nuclei, objects were defined in images selected for analysis with Image-Pro Plus version 7.1, using a modified method of DeCoster et al. (2010). Briefly, raw pseudo-colored TIFF images were exported to Image-Pro Plus and the

‘count bright objects’ was selected in the count/size menu which measured all individual or populations of cell nuclei positive for Hoechst stain and neuronal perikaryon positive for NeuroTrace™ fluorescent dye. The ‘watershed split’ and ‘split object’ functions were used to separate nuclei/cell bodies, which overlapped each other. Nuclear outlines not properly identified by the software were retraced manually using the ‘draw/merge objects’ function and included in the analysis. It was confirmed by visual inspection that these objects were indeed condensed nuclei that retained an outline too faint to be identified automatically. To avoid counting nuclei or cell bodies touching the edge of the counting field, the ‘toggle objects on/off’ function was employed. To remove these ‘toggled off’ objects from the analysis, the ‘delete hidden (off) object’ was selected, followed by the ‘count’ function. In addition, this technique is useful in isolating and removing potential artifacts that may produce false-positive events, such as apoptotic bodies or out of focus nuclei, which especially present difficulties, depending on the thickness of the section (DeCoster et al., 2007). A minimum of 360 cell nuclei was counted per section. All morphologic parameters were quantitated using Image-Pro Plus software. Fluorescent labels were chosen over traditional chromatic stains for quantification because of their higher signal-to-noise ratio provides superior digital image analysis of morphological parameters (DeCoster et al., 2007).

.

Myelin Content Analysis: Photometric estimation of myelin intensity to determine the Content of myelin was carried out on paraffin sections of the brain. We utilized the intensity function of (Image-Pro Plus version 7.0) to automatically measure the image intensity for the green fluorescence of fluoromyelin. Summary statistics for each image was computed automatically and then transferred to an excel spreadsheet.

Nuclear Area Factor (NAF) Computation: Photomorphometric estimation of nuclear changes in non-neural cells to determine apoptotic change was carried out on paraffin sections using Image-Pro Plus version 7.0. Neuronal cells, Hoechst labeled nuclei that were NeuroTrace^{TM} (Neurons) positive (H^+NT^+) were 'toggled off' (see image analysis) to remove them from the analysis. Following which nuclear area and roundness of non-neuronal cells (H^+NT^-) were determined by selecting the measure function. The resulting data for each image was transferred to an excel spreadsheet, after which the nuclear area factor (NAF) for each nuclei was computed (DeCoster et al., 2007; Affifi et al., 2012) as the product of the nuclear area and roundness (roundness*area).

Tissue Processing for Transmission Electron Microscopy (TEM): To evaluate ultrastructural changes, 1-mm^3 blocks of tissues were immersed overnight in 2.5 % glutaraldehyde at 4^{o}C, post-fixed in 1 % osmium tetroxide, dehydrated in ascending concentrations of ethanol, cleared in acetone and embedded in EMbed-812 (Electron Microscopy Science). Thick sections (1.0 µm) were cut using an ultra-microtome (Reichert Ultracut E, Austria) followed by staining with toludine blue.

EM Stain Ultrastuctural myelin changes were determined according to standard TEM protocols as previously described in our laboratory (Reuhl et al., 1993). Briefly, thin

sections (60-65 nm) of cerebral brain tissue embedded in EMbed-812 were mounted on EM grids, stained with uranyl acetate and lead citrate prior to micrographic analysis. Ultrastructural images were viewed using a Zeiss EM 10 CA transmission electron microscope (Carl Zeiss GmBH, Germany). Electron micrographs were obtained with a plate camera attached to the Zeiss electron microscope. EM negatives were scanned (Minolta, Dimage Scan II, Japan) and saved as digital images. All digital images in the study were routinely saved on to the hard drive of the computer and on recordable compact discs (CD-R) as a backup.

1.4.4 Results

1.4.4.1 Clinical Effect of AMB and AME

AME-treated mice (5 mg/Kg b.w) showed no signs of clinical intoxication after 1-month of injections, contrasting similarly treated AMB-mice. Data in table 1.4a shows AMB induced lethargy and weight loss in the animals, while AME caused a weight gain. Suggesting AME may be less toxic than AMB after repeat dosing.

Table 1.4B shows that AMB's higher toxicity relative to AME was more pronounced after 2-months of injections. One out of the four AMB-mice died during the injections. AMB mice also lost weight and were often lethargic. In contrast, all AME mice appeared clinically normal and gained weight. Although no AMB-mice during a subsequent 3-month observation (with cessation of treatment) died, clinical intoxication

persisted with further weight loss (Table 1.4B (b)). In sharp contrast, AME-mice on average gained weight with no evidence of clinical intoxication, consistent with the observed trend of a reduced toxicity of AME relative to AMB.

The reduced toxicity of AME was also apparent after chronic administration (7 mg/Kg b.w). None of the AME-mice died after 5-monthsof alternate day injections; rather they gained weight (67 %), similar to the glucose controls (Table 1.4C). Glucose was used as a control because it was used as a vehicle to reconstitute the test compounds. Signs of clinical toxicity were not obvious in both vehicle control and AME-mice throughout the study. This implies that cumulative doses of highly purified AME may not be toxic when given at clinically attainable doses.

1.4.4.2 Histopathologic Comparison of AME and AMB

To evaluate the apparently increased toxicity of AMB over AME, we compared both drugs by histopathologic techniques. We examined cerebral brain sections of adult mice for myelin loss and nuclear morphological change in cerebral white matter after administration of AMB and AME for 1-, 2-, and 5-months. Paraffin embedded sections of adult Balb\c mouse brain were obtained as described in Materials and Methods. Representative sections were stained with chromatic dyes; hematoxylin counterstained with eosin, and the myelin specific luxol fast blue dye counterstained with the Nissl substance stain cresyl echt violet (see material and methods). In addition, we used computerized fluorescent quantitative microscopy employing fluoromyelin to label

Table 1. 4 Clinical Signs

Following (A) 1-month, (B) 2-months of AME and AMB administration at dose of 5 mg/Kg b.w, AMB mice lost weight. (C) 5-months of AME treatment at a higher dose of 7 mg/Kg b.w did not lead to weight loss in AME mice. Due to insufficient animals (A) n=1 and (B) n=1 for controls, statistical analysis could not be applied to the data. For a similar reason, AMB was compared alone (no AMB group) to controls.

A				
1-month exposure	Weight Change (% of pretreatment wt)	±SD	Lethargy/irritation	Death/ Total animals tested
Controls	6.2	-		0/1
AME	6.6	-		0/1
AMB	-11.4	-	+	0/1
B(a)				
2-month exposure				
Controls	3.4	-		0/1
AME	6.3	1.375654		0/4
AMB	2.6	4.901683	+	1/4
B(b)				
3-month drug-free				
Controls	38	-		0/1
AME	10	2.342328		0/4
AMB	-8.8	5.567506	+	0/3
C				
5-month exposure				
Controls	57.2	±10.176120		0/4
AME	61.9	±15.515188		0/4

myelin and NeuroTrace™ to label neurons, Hoechst 33342 was used as a counter stain for nuclear chromatin.

1.4.4.2.2 Effect of AME and AMB on Cerebral Myelin

Light microscopic examination of formalin-fixed brain sections of mice treated with AMB for a month revealed a diffuse myelin injury in the cerebral white matter. These lesions are evident in hematoxylin and eosin stained sections (Figure 1.9). Figure 1.10 shows hypochromatic areas in the cerebral white matter that was consistent with the loss of myelin. A similar pathology was not observed in the AME-brains (Figure 1.10). Further analysis (Figure 1.11) revealed areas of diminished fluoromyelin intensity, reflecting myelin loss. Neurons completely devoid of myelin appear as red dots or yellow for those with intact myelin.

Following 2-months of alternate-day injections of AMB and 3-months of cessation of treatment, myelin lesions were observed on routine H/E histological stains (Figure 1.12). However, use of specialized myelin specific stains such as LFB (Figure 1.13) and fluoromyelin (Figure 1.14) revealed receding myelin or myelin stripping of neurons with degenerating neurons in the AMB-brains. These lesions were not confined to a particular region, but appear diffusely in the sub cortical white matter. Similar lesions in the same region were absent in AME- and control-mice.

Figure 1. 9 White matter vacuolations occurs with amphotericin B exposure

Images below show light microscopic analysis of paraffin sections of mouse cerebral white matter after staining with hematoxylin and eosin following 1-month exposure of adult Balb/c mice to AMB and AME. (A, B) AMB, (C, D) AME treated group and (E, F) vehicle controls. Black arrows depict myelin stripping of neurons. Scale bar is 200 µm.

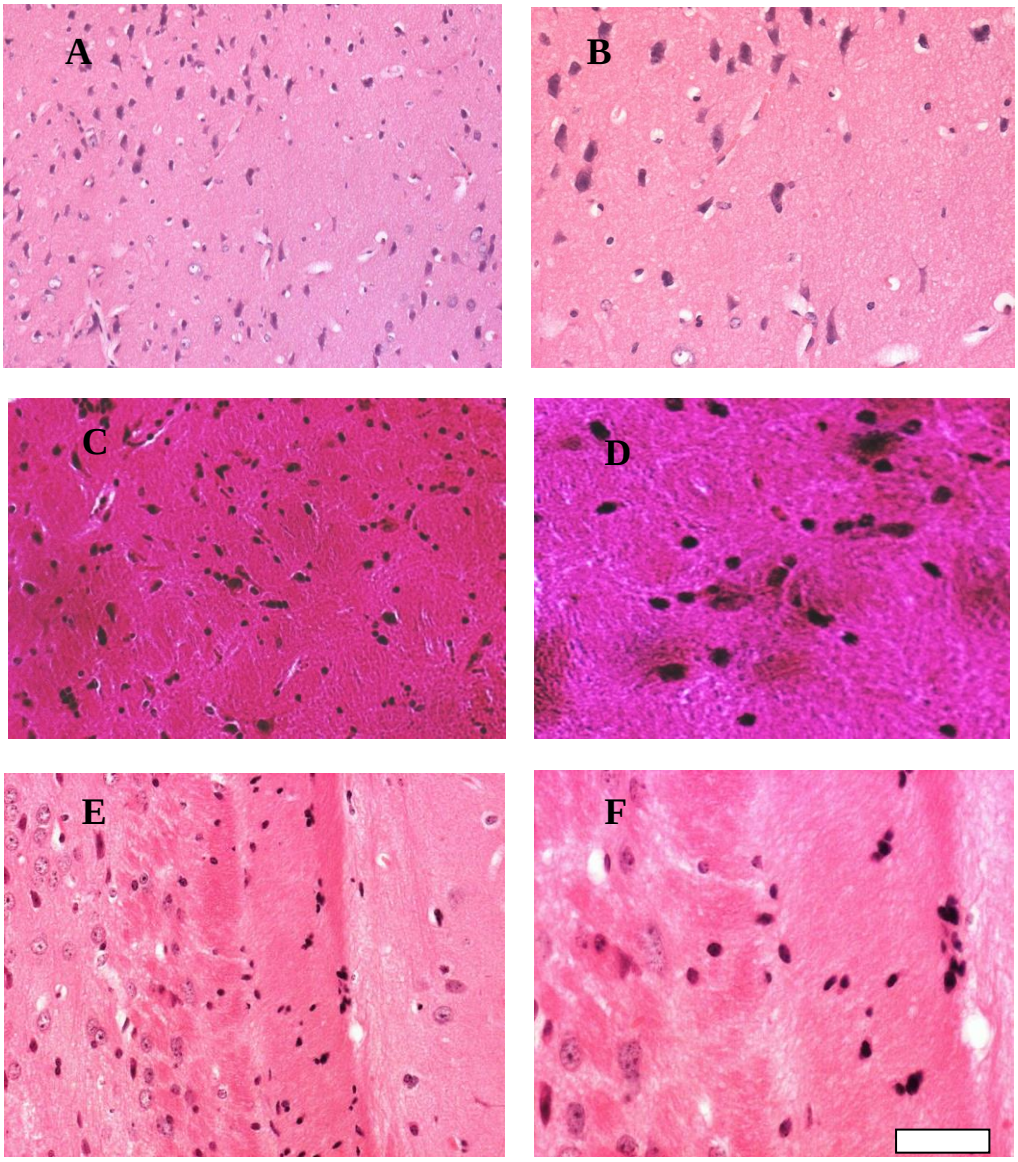


Figure 1.10 Receding central myelin is observed after AMB treatment

Representative paraffin sections of mouse cerebral white matter analyzed by light microscopy after the special myelin stain luxol fast blue was counter-stained with the chromogenic Nissl stain cresyl violet demonstrated no myelin deficits in (A, B) control animal or (C, D) adult Balb/c mice treated i.p with 5mg/kg of AME or AMB (E and F) every other day for 28 days. Magnification is 20x (A, C) and 40x (B, D). Scale bar is 100 µm.

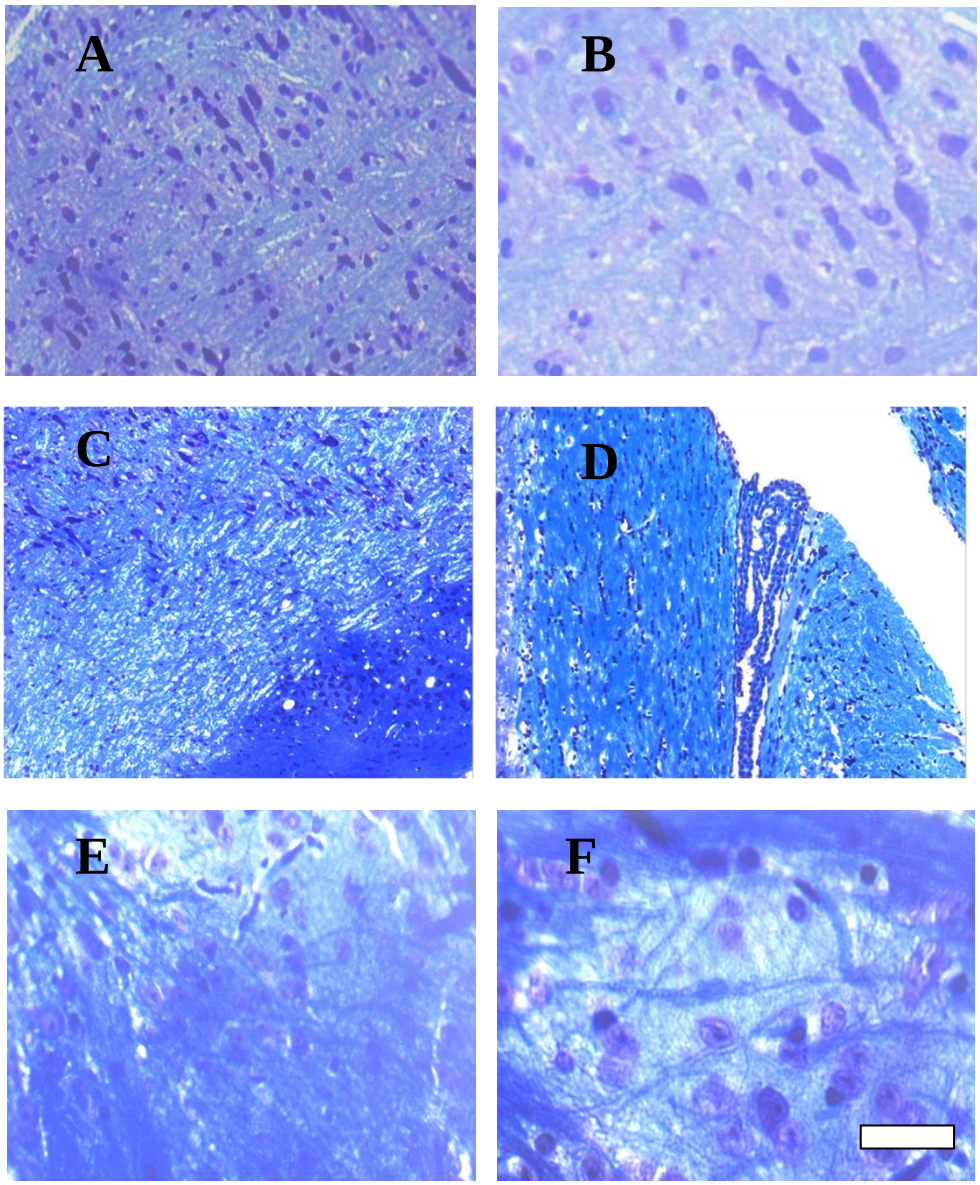


Figure 1.11 AMB elicits myelin changes in central white matter of mice

(Panels A - D) No indication of central myelin damage was visible after an alternate day dose of 5 mg/Kg AME was given intraperitonealy to adult mice for 1-month. The lack of lesion was evident after analysis with a combination of three different fluorogenic probes; fluoromyelin (A; green), NeuroTrace (B; red) and the DNA dye Hoechst 33342. (Panels E - H). However, examination of brain sections of mice receiving 5 mg/Kg AMB dosed every 2 days confirmed the presences of myelin stripping evidenced by denuded areas (H; red arrows), silhouette of dead oligodendroglia or neurons (H; indented white arrows). (Panels I-L) the controls were free of pathology. Scale bar is 100 µm.

AME	AMB	CONTROL

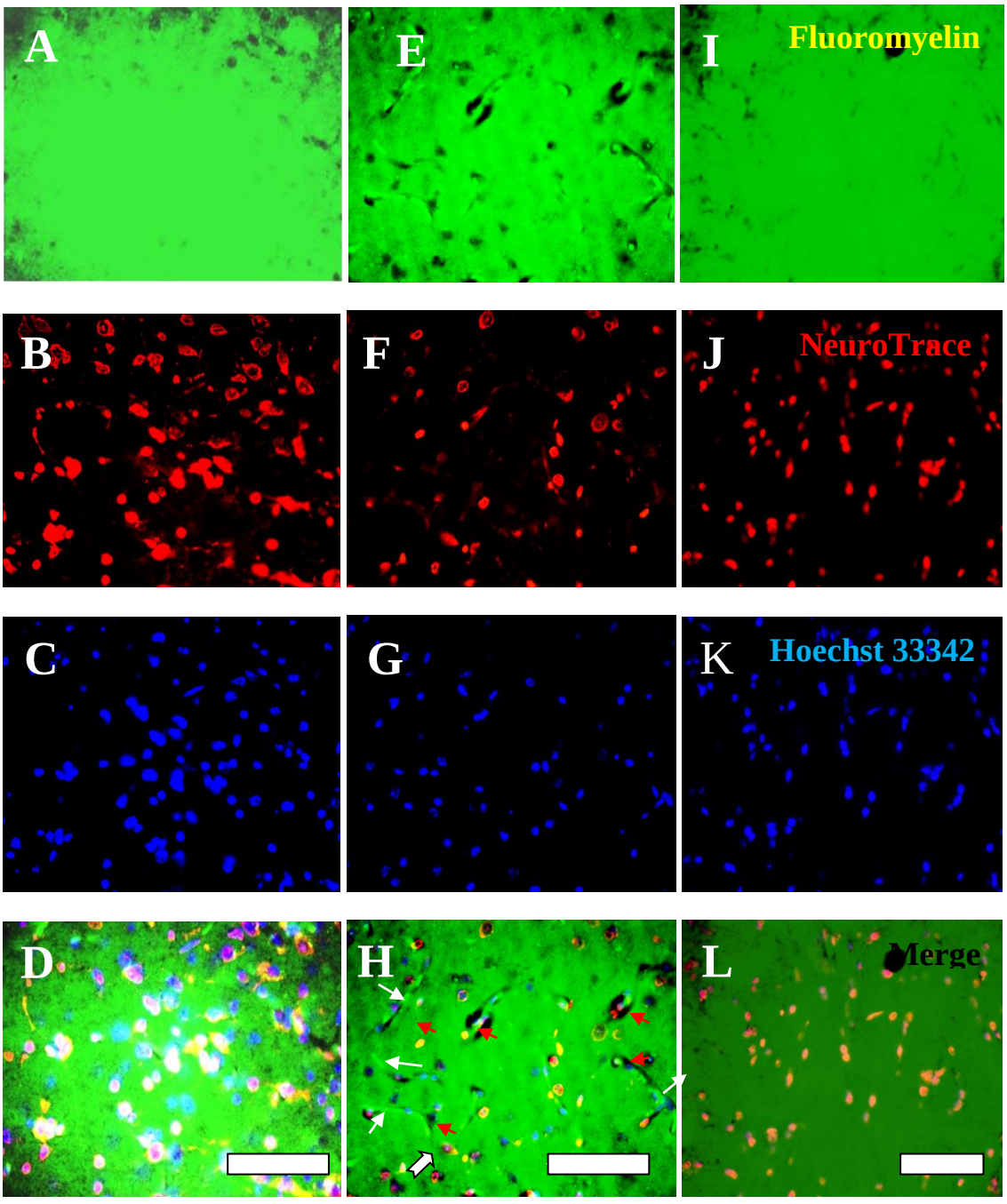


Figure 1.12 Myelin lesions assessed by H/E staining

Myelin lesions are apparent following an observation period 3-months post treatment with AMB (5 mg/Kg) that was given on alternate days for 2-months (A, B) outline by black arrows. Similarly treated AME mice (C, D) had no lesions much like the dextrose control group (E, F). Scale bar is 200 µ.

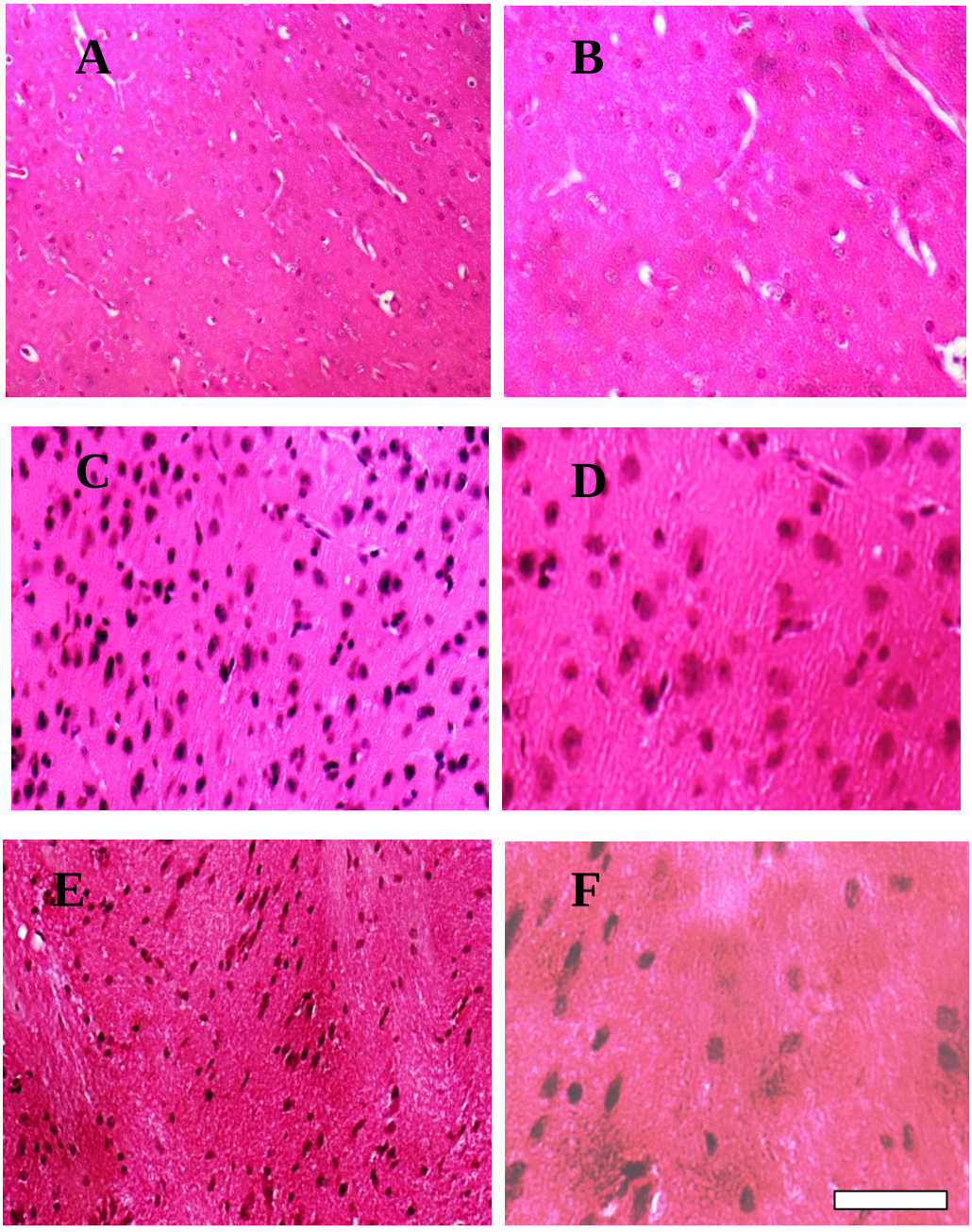
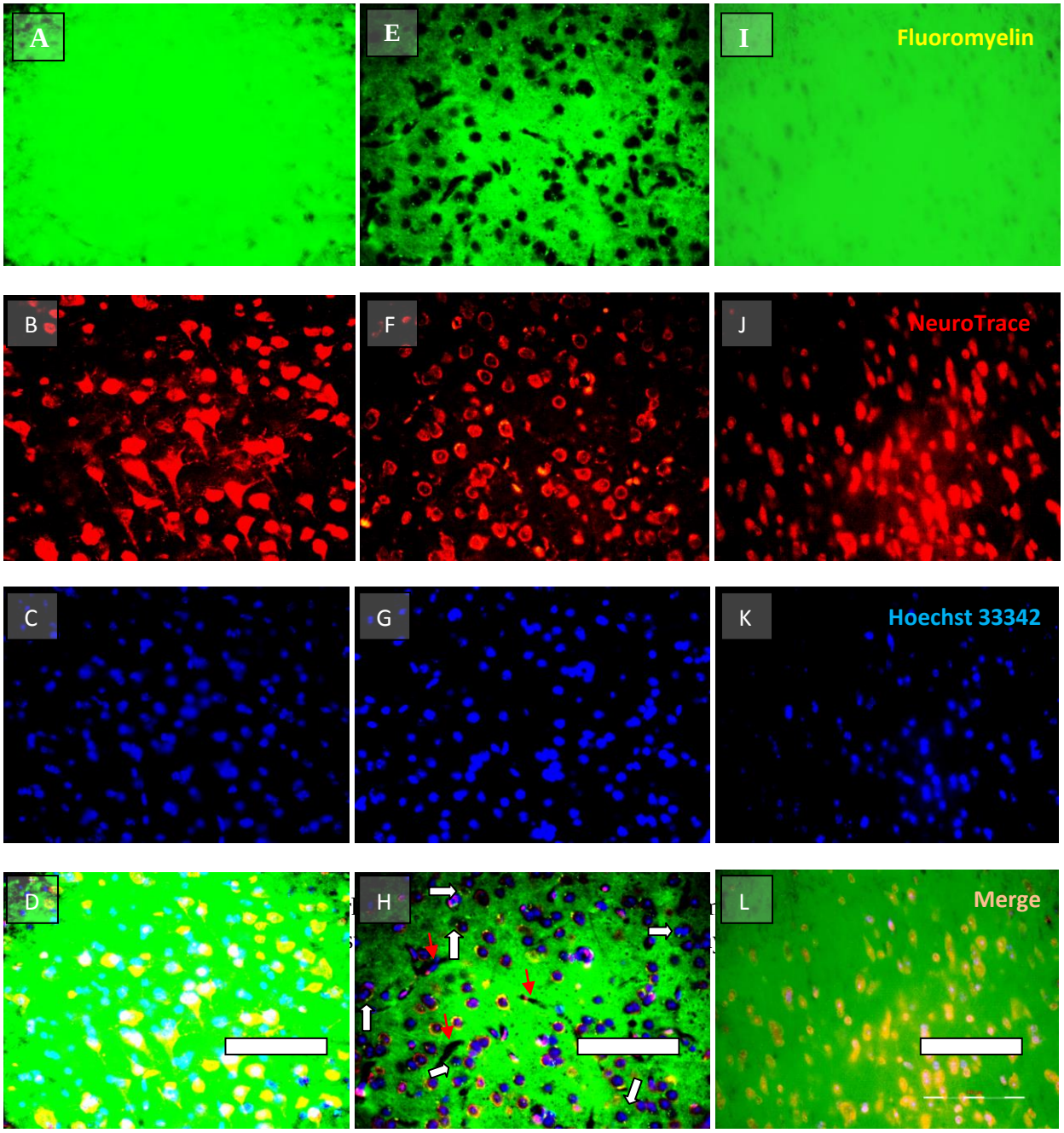


Figure 1.13 Fluoromyelin analyses for myelin loss

An average cumulative dose of 1.1 mg of AME was given intraperitonealy to adult mice for 2-month followed by an observational period of 3-months. Myelin or neuronal cell injury was not apparent after fluorescent microscopic analysis of paraffin sections of AME-brains (A, B, C, D) labeled with the triple stain of fluoromyelin (green), NeuroTrace (red) and the DNA dye Hoechst 33342 (blue). In Contrast, it appears that after allowing an observation period of 3-months post treatment, the group of adult mice treated i.p with AMB (5mg/Kg) for 2-months developed neuropathology, evidenced by prominent myelin stripping and vacuolation (red arrows) as seen on micrographs E and H. In addition, there appears to be an invasion of the vacuolated areas by $Hoechst^{+}$ cells (indented white arrows) that may have engulfed myelin debris (highly fluorescent green dots) and in some cases Nissl granules. (Panels I-L) represents the controls. Scale bar = 100 µ

AME	AMB	AME


A
E
I
Fluoromyelin
B
F
J
NeuroTrace
C
G
K
Hoechst 33342
D
H
L
Merge

1.4.4.2.3 Quantification of Myelin Loss

To reduce investigator bias while evaluating myelin deficits due to either AME or AMB, we employed automated image analysis methods to determine fluoromyelin intensity by light microscopic fluorescence methods. These *in vivo* experiments served to provide feasibility on the automated method of measuring fluorescent intensity using membrane permeant fluorescent probes in our main *in vitro* study. For each mouse brain, we labeled several six μm paraffin sections of cerebral white matter with the green fluorescent myelin dye, fluoromyelin, obtained digital pictures and analyzed them using image ProPlus 7.0 (Kanan et al., 2006; see Material and Methods).

Photometric analysis of the fluorescent images (Figure 1.11) from our exploratory study demonstrated the lower myelin content of AMB brains as depicted by diminished fluoromyelin intensity after 1-month of alternate day injections (Table 1.5). In contrast, AME brains did not show diminished myelin intensity. Although our data suggests a loss of myelin in the white matter of the sub cortical cerebral region of AMB-treated mice, this inference cannot be made due to insufficient number of animals (n=1).

Similarly, inference cannot be drawn with the myelin deficits recorded in AMB-treated mice (Figure. 1.13) after 2–months of treatment (Table 1.6) because of insufficient number of controls (n=1). Nevertheless, diminished myelin content of similarly treated AME-mice was not apparent.

Table 1. 5 Myelin quantification after 1-month alternate day injections of AMB and AME (a pilot assessment)

The results below represent a pilot assessment of the semi-automated quantification of fluorescent intensity using the myelin specific fluoromyelin dye. Note that the standard deviation (SD) for the average pixel intensity is per animal and not the average pixels intensity of a treatment group. This pilot assessment allowed us gain preliminary data for feasibility on use of the imaging analysis software for the main *in vitro* study.

Treatment	Number of Animals (n)	Number of fields counted/brain	Average Myelin intensity (Pixels)	SD/animal
Controls	1	6	102.8822	±24.4994
AME	1	5	94.7431	±7.70034
AMB	1	6	46.60069	±11.84069

Table 1. 6 Myelin quantification following 2-month alternate day injections followed by 3-months cessation of treatment

The results below represent a pilot assessment of the semi-automated quantification of fluorescent intensity using the myelin specific fluoromyelin dye. The standard deviation (SD) for the pixel intensity (average) for the vehicle control is per animal (n=1) and does not represent a true standard deviation. For the AME and AMB treatment groups (n= 4 and 3 respectively) the average myelin pixel intensity for the group was computed as mean +/- SEM. However statistical inference could not be made due to low number of animals in the control group.

Treatment	Number of Animals (n)	Number of fields counted/brain	Average Myelin intensity (Pixels)	
Controls	1	6	119.4181	±26.47821 SD/mice
AMB	3	6	40.66858	±7.751891 (SEM)
AME	4	6	94.12634	±16.04168 (SEM)

1.4.4.2.4 Nuclear Morphology Assessment

To determine whether nuclear morphology of cells in the cerebral white matter exhibited apoptotic change, we examined for $H33342^{+}$, a non-specific fluorescent membrane permeant nuclear dye that stains all nuclei blue. We also examine for NeuroTrace, a membrane permeant fluorescent probe that stains Nissl substance of neurons (Kannan et al., 2010). Co-localization of $H33342^{+}$and NeuroTrace was examined by fluorescence photometry. Nuclear morphological analysis was obtained from parallel fields where diffuse demyelination was observed. The primary purpose of this experiment was to lay the ground work on the feasibility of using semi-automated techniques to quantify changes in nuclear morphology with regards to the main cell culture studies.

In the brains of mice treated with AMB for a month, we observed nuclear morphological alterations in the brain sections (Figure 1.14). In contrast, the cerebral white matter of the AME-treated brains and vehicle controls had few apoptotic nuclei on microscopic examination of H33342 labeled nuclei. Although the single animal (n=1) observation of apoptosis in the brain gives clues that neuronal apoptosis may occur in healthy mice after AMB administration, the number is insufficient to draw statistical inference.

Likewise, AMB-brains exposed to 2-months of alternate day injections (Figure 1.14) appear not to follow this trend as we observed much less apoptotic nuclear change in the same subset of non-neuronal cells. However, it is difficult in this study to

determine the magnitude of difference in apoptotic change between the two experiments because of insufficient numbers of animals, thus, prompting quantitative estimation in cell culture models.

1.4.4.2.5 Quantifying Nuclear Alteration in Cerebral White Matter of Mice

Automated image analysis or Image cytometry (ICM) was used to extract quantifiable histopathologic information on the nuclear morphological changes observed in AMB and AME treated brains of adult mice. Images of six non-overlapping fields of 6-micron sections of each adult mouse cerebrum were used for analysis. After a month of AMB, the NAF values for AMB treated brains shows a reduction (Table 1.7). A reduction in NAF value is reflective of apoptotic nuclear change. Comparison with controls was not carried out due to low number of both treatment and control animals (n=1). AME appears to have an NAF that was higher than that of AMB. This data provides evidence of apoptotic change of non-neuronal cells in areas of demyelination. However, the data was not amenable to statistical analysis due to low number of animals (n=1).

This preliminary study also confirms the feasibility of using semi-automated image analysis and such parameters as the nuclear area and round NAF to quantify the morphology of normal and abnormal nuclei. This assessment was not carried out in the 2-month study as the preliminary data obtained provided feasibility and proficiency for use of the method in the main *in vitro* study.

Figure 1.14 AMB causes apoptotic nuclear change in cerebral white matter of mice

Below are representative images of Hoechst nuclear dye labeled mouse brains after a month of AME and AMB administration. Arrows indicate condensed chromatin, reflective of apoptotic nuclei. Micrograph A is the glucose controls, B is AME and C is AMB treated cerebral white matter sections. Scale bar is 100 µm.

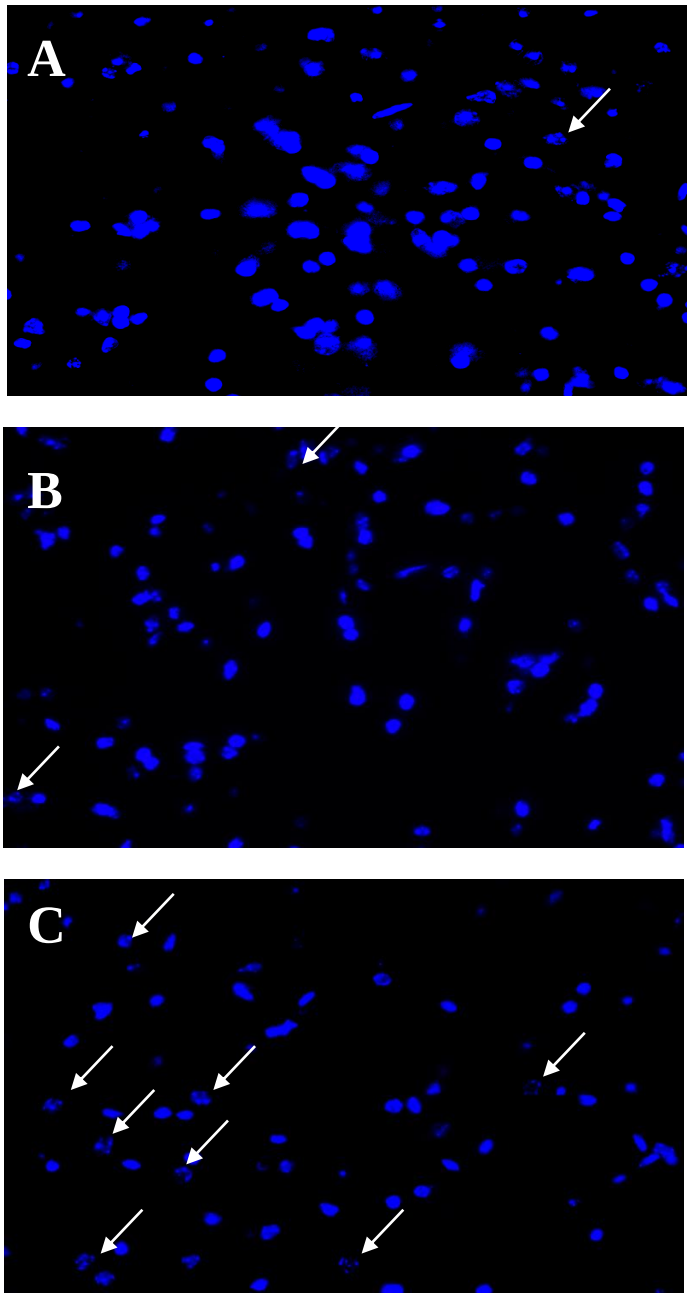


Table 1. 7 Quantification of Apoptotic nuclei

The table below summarizes the effects of AMB and AME on the nuclear area factor (NAF) of non-neural cells in the cerebral white matter of mice. Note that the nuclear area factor (NAF) was computed for each nucleus that was NeuroTrace™ red negative (non-neuronal cells) and expressed as the average SD of each animal (n=1) and not the experimental group and thus, does not represent a true SD.

Treatment	Number of fields counted per brain	Mean NAF /animal	SD/per animal
Control	5	33.2	±5.522056
AME	5	31.1	±8.151946
AMB	6	21.4	±5.430702

1.4.4.2.6 Effects of Chronic AME Administration

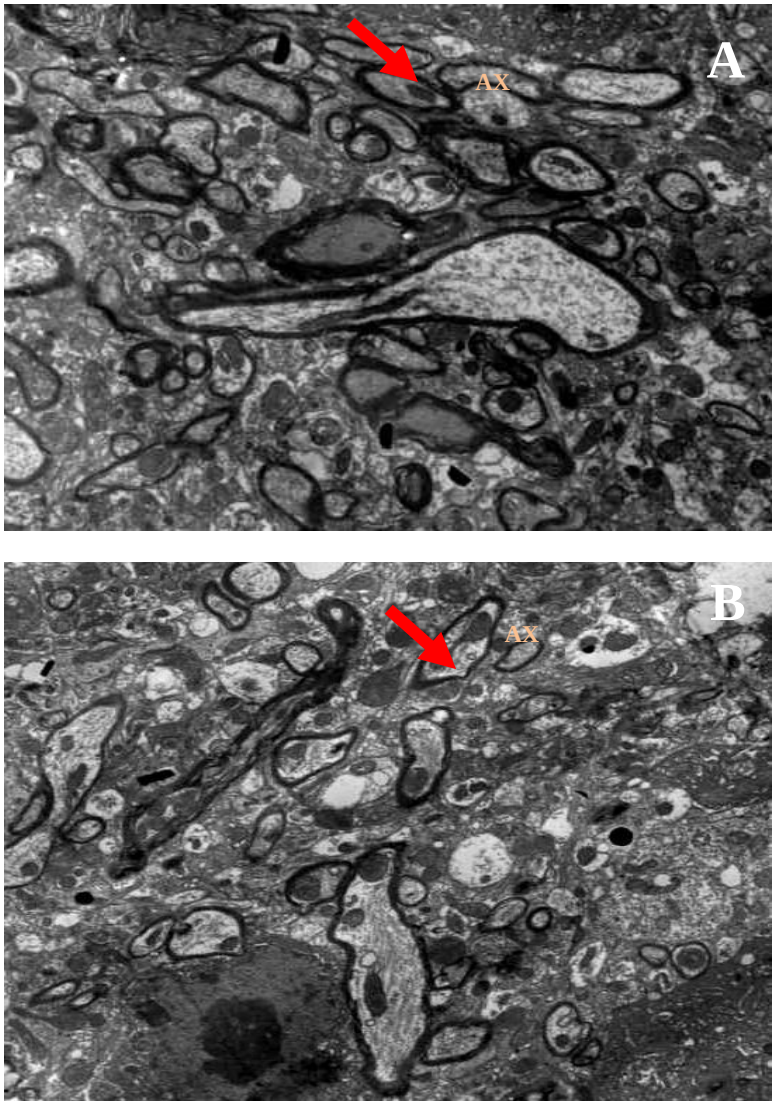
The link between chronic exposure to pure AME preparations and demyelination was evaluated. We chose this study because no appreciable myelin defects were noted in AME mice acutely or sub-chronically, and it is felt that cessation of treatment might have permitted recovery of any mild, AME-induced injury. Furthermore, owing to its favorable therapeutic index (Schaffner and Mechlinski 1972; Mechlinski and Schaffner, 1972; Hoeprich et al., 1988) it is possible that the AME dose we used (5 mg/Kg b.w) although clinically relevant, was not acutely toxic. To investigate these possibilities, we administered i.p AME (7 mg/Kg b.w) at a dose higher than that used clinically but much lower than the LD_{50} of 86 mg/Kg b.w over 5-months to adult male mice with glucose controls (see Materials and Methods).

Despite receiving an average total of 12.5 mg of AME, analysis by electron microscopy examination confirmed absence of significant myelin pathology (Figure 1.15) in the AME brains, or 5% glucose control-brains. Again, due to insufficient test animals, a comparative study including AMB or AMB as a contaminant was not undertaken; nonetheless, these chronic comparative assessments will help improve the current study as it will mimic the long duration of treatment expected in clinical therapy.

Kidneys were examined as a positive control for AMB content of the AME preparation. There was no real toxicity; however, had there been significant AMB contamination, massive renal injury would have resulted earlier from its effects.

Figure 1.15 Amphotericin methyl ester does not elicit demyelination after chronic use

(A) Although adult mice received an average total of 12.5 mg of AME over an extensive period of 5-months via the intraperitoneal route, no ultrastructural myelin lesion was observed on electron microscopic examination of central white matter similar to (B) the control animals. The representative electron micrographs above illustrate the normal morphological relationship between axons and compact myelin. The arrows indicate normal compacted myelin around the axon (Ax). Magnification is 2000x.



1.4.5 Discussion

The present study aimed to examine the relative neurotoxities of AME and AMB *in vivo* and provide feasibility to carry out a mechanistic assessment to address their neurotoxicity potential in live-cell culture systems. The study also aimed to Preliminary observation of increased AMB-induced lethargy, weight loss and death relative to AME in adult mice, implied an increased general toxicity for AMB. Histological assessment of sections of cerebral white matter demonstrated a trend that sub-chronic AMB administration but not AME leads to myelin injury (Leukoencephalopathy) that is not easily reversed. Indeed, chronic treatment, even with supratherapeutic doses of AME alone did not result in significant neuropathology. In addition, our observation suggests that prolonged use of AMB and not AME results in irreversible injury to myelin in sub-cortical white matter. Data from previous studies associate AME with myelin injury and AMB as non-neurotoxic. However, recent data argue against AMEs reputed neurotoxicity and demonstrate that AMB is neurotoxic. The evidence of features found in areas of demyelination that were consistent with apoptosis (Varlam et al., 2010) implying that AMB may act via apoptosis to cause neurotoxicity. The feasibility of quantitating morphometric changes employing the nuclear area (NAF) assessed by a semi-automated method was evident in this preliminary study. There was a trend of AMB causing a reduced NAF. AME did not induce nuclear lesions.

Intraperitoneal administration of alternate day AME and AMB led to a higher general toxicity of AMB-treated after 1-month. Furthermore, the trend of increased AMB-induced general toxicity relative to AME in adult mice was observed after 2-

months of alternate day injections. The reason for the continued general toxicity long after treatment stoppage was unclear. In contrast, the lower general toxicity of AME following chronic administration was reflective of its lower toxic disposition (Schaffner and Mechlinski, 1972; Reuhl et al., 1993). Unlike with AMB, AME revealed chronic kidney damage (data not shown).

Histological evidence revealed that sub-chronic AMB administration but not AME leads to myelin injury (Leukoencephalopathy). This toxicity manifested as diffuse myelin pallor (or loss) with myelin retraction and axonal degeneration following a month of exposure. These myelin lesions were present in the cerebral white matter of the AMB-mice, resembling lesions reported in AMB-treated rats (Reuhl et al., 1993). Further, AMB induced myelin loss is not readily reversed on cessation of treatment (Gold et al., 1989; 1990; Reuhl et al., 1993) after 2-months of administration followed by 3-months of observation. Indeed, very little evidence of myelin repair (thinning myelin profiles, oligodendrocytes proliferation) was noted by either light or electron microscopy.

AMB induced central demyelination in our preliminary studies. However, additional studies using larger numbers of samples in the 1-month study and the 2-month administration with treatment cessation are required to gauge if the pathologic changes observed were significant. Consistent with our hypothesis, AME dosed similarly as AMB did not cause remarkable demyelination. These differences were not a species difference, as most myelin toxicants such as hexachlorophene, triethyl tin, etc show similar effects across species (Morell and Toews, 1996).

These differences may be accounted for by the physiochemical differences between the two drugs. For example, the increased affinity of AMB for lipid membranes over AME may suggest why myelin appears to be more sensitive to AMB relative to AME, since myelin membranes are rich in cholesterol (Gadebusch et al., 1976). In addition, considerable pharmacokinetic differences between the two compounds and a striking difference in their ionic states at neutral pH may contribute to the observed variability in toxicity.

Cass and co-authors (1970) provide an additional explanation for lack of myelin lesions by high purity AME relative to AMB. They suggested that membrane pore formation in cholesterol membranes; these membranes for membrane exposed to AME but not AMB can reverse a generally accepted mode of AME action. This means that a membrane repair mechanism may exist in cholesterol containing membranes for AME that can return the membranes to its normal state after permeabilization.

It is unclear if residual AMB has functional effects on metabolically active cells in the brain. Activation of reactive microglia for instance, may lead to release of immune and oxidative toxic factors that might in turn continue to incite injury (Lull and Block, 2010) depleting oligodendrocytes as seen in dogs treated with impure AME (Ellis et al., 1988). Alternately, if an irreversible primary axonopathy as reported by Gold et al (1989, 1990) occurs with AMB treatment, then it is likely that myelin failure will also occur since myelination requires a viable axon. Nevertheless, the clinical significance of an irreversible AMB-induced myelin injury is the risk of residual pathology that may manifest as neuropathological and neuropsychiatric dysfunction (Winn et al., 1979;

Weddington, 1982; liu et al., 1990). A functional observatory battery may provide a non-invasive approach to test for residual pathology.

Our preliminary data showed AME was not toxic even after chronic dosing. This suggests AME may not induce neuropathology consistent with previous studies (Reuhl et al., 1993; Racis et al., 1990). Alternately, it is possible the current study failed to detect signals of AME-induced myelin pathology. One way to improve this study may be to experimentally open the blood brain barrier using osmotic diuretics such as inulin and mannitol before administering AME. This would enable AME (and AMB) easy access into the brain and could also mimic the fungal disease states (BBB permeability) for entering the CNS. Our preliminary inquiry has provided feasibility for use of automated quantification methods employing non-toxic fluorescent molecular probes that can be adapted easily to subsequent live-cell studies. These techniques can also be applied to future studies with an open BBB model.

Because some of the side effects of AME and AMB are reversible, and because apoptosis has been reported in kidney cells following exposure to AMB (Varlam et al., 2010), we conjectured that AMB and not AME might induce apoptosis in our mouse brains at therapeutic doses (5 mg/Kg/b.w). Although our current study was not amenable to statistical analysis, we observed on fluorescent microscopy after a month of of injections, a nuclear morphologic alteration consistent with apoptotic change, in AMB-mice. The changes were manifest as nuclear chromatin condensation, margination, fragmentation, and intact nuclear membranes consistent with apoptotic change (Buntinix et al., 2004). These lesions were localized mainly to a subset of non-neural cells in areas of demyelination; however, similar lesions were absent in AME treated-mice.

Because non-specific nuclear fragmentation may mimic apoptosis (Varlam et al., 2010), we further analyzed brain sections using computerized fluorescent quantitative techniques. The computed nuclear area factor (NAF) result followed the observed trend, signifying that perhaps, apoptosis may mediate some aspects of AMB-induced myelin injury. To examine this hypothesis *in vitro,* the NAF allows for a rapid non-invasive assessment (screening) of nuclear changes that could reflect apoptotic change. This current *in vivo* preliminary assessment has provided proficiency in the use and feasibility of the cytometric parameters of nuclear area and roundness for the efficient, reliable and rapid computation of the NAF.

This current study has also demonstrated that highly purified AME may not be neurotoxic even at supra-therapeutic doses when chronically administered. In stark contrast, a smaller dose of the commonly used AMB formulation (Fungizone®) caused myelin damage sub-chronically. In addition, based on our preliminary observation, it is tempting to speculate that AMB in part, may be neurotoxic by inducing apoptotic change. Furthermore, the study provides feasibility to carry out an *in vitro* mechanistic inquiry using non-invasive and semi-automated quantification techniques.

Though the response observed in this study follow a pattern consistent with recent literature, caution should however be exercised when interpreting the preliminary results due to the unavoidable limitations posed by small (insufficient) sample size of animals used. To better characterize the mechanism for the toxicity, an *in vitro* set of studies was carried out to better address these questions.

Chapter 2

2 *In Vitro* Toxicity of Amphotericin B and Amphotericin B Methyl Ester to Oligodendrocytes

2.1 Abstract

This chapter compares effects of Amphotericin B (AMB) exposure on oligodendrocytes with those of its methyl ester derivative, AME. Specifically, the mode of cytotoxicity, believed to result from plasma membrane permeabilization, was characterized by morphological and biochemical analysis. AMB is widely used in the treatment of chronic systemic fungal disease, but prolonged treatment often results in a dose-limiting nephrotoxicity. AME, a polyene macrolide with significantly less toxicity than AMB and comparable antifungal activity, was introduced in the late sixties. However, its clinical use was terminated following reports that its use caused leukoencephalopathy. Recent studies from our lab using highly purified AME suggest that the reported neurotoxicity resulted from contamination by AMB, resulting in oligodendrocyte and myelin injury. To test this hypothesis, immortalized human oligodendrocyte cells (MO3.13) were treated with 0.5-30 µg/ml AMB and AME. A quantitative dose-response assessment using dye exclusion methods was used to assess cytotoxicity. The lowest concentration of AMB to induce cell death was 1 µg/ml and the lowest concentration of AME to have comparable effects was10 µg/ml. A mixture of 1

µg/ml AMB and 9 µg/ml of AME appears to cause cell fragmentation, contrasting 9 µg/ml of AME alone. Hoechst labeled nuclei revealed a significantly diminished nuclear area factor (NAF) following AMB treatment at 1 µg/ml, suggesting apoptotic change but these measures were unaltered by AME concentrations below 10 µg/ml. These data suggests that AMB, but not AME, is lethal to oligodendrocytes by inducing cytotoxicity. In addition, apoptosis may contribute to AMB-toxicity.

2.2 Introduction

Amphotericin B methyl ester (AME) is a water-soluble analog of Amphotericin B (AMB), the commonly used antibiotic for serious fungal disease. It is generally agreed that AMB and other macrolide antibiotics are cytotoxic to fungi by means of their affinity for the unique fungal membrane ergosterol (sterol), subsequently inducing formation of plasma membrane ionophores with trans-membrane fluxes of cations (particularly K^+) and consequent membrane depolarization. AMB's mammalian toxicity is attributed to its interaction with membrane cholesterol, also a sterol. AMB remains the drug of choice for chronic systemic fungal disease, but patients often suffer severe side effects, particularly a dose-limiting nephrotoxicity, underscoring the need for improved antifungal therapies (Racis et al., 1990).

AME, an improved analog of AMB developed by Mechlinski and Schaffner (1972), retains full antifungal activity and more favorable pharmodynamics. Unfortunately, clinical use of AME was discontinued due to the appearance of

leukoencephalopathy (myelin damage) in patients receiving experimental treatment for disseminated *C. immitis* infection. This observation was complicated by the fact that the formulations used clinically were extensively contaminated with AMB. Subsequent studies using pure AME formulations have demonstrated negligible neurotoxicity. Nevertheless, AME remains an embargoed drug.

AMB can be toxic to the brain. Prior experimental animal and *in vitro* studies in our lab, alongside the cell culture experiments by Racis and coworkers (1990), demonstrate that oligodendrocytes the myelin producing cells of the CNS, which are characteristically rich in cholesterol, may be a direct target of AMB and impure AME formulations (Reuhl et al., 1993). In support, Gold and coworkers (1989; 1990) provided evidence that AMB caused PNS myelin toxicity, associated with Schwann cell death (Reuhl et al., 1993). This suggests that leukoencephalopathy reported in earlier studies may have resulted from AMB contamination (including multi-methylated AMB derivatives) rather than AME itself.

The formation of membrane pores and subsequent potassium efflux by AMB has been shown to induce cytotoxicity and apoptosis in some non-CNS cell types (Markund et al., 2001); however, very little is known about the impact of AMB in promoting oligodendrocyte cell death. This raises some critical questions concerning the mode of AMB and AME actions on this cell type: Does AMB/AME induces a change in cell membrane permeability of mature oligodendrocytes? Do both drugs inhibit oligodendrocyte viability, or is toxicity selective for one? Do these cells die by apoptosis *in vitro*?

In this chapter, we address these questions and test the hypothesis that AMB, but not pure AME, at therapeutic concentrations has direct toxic effects on oligodendrocytes, using the immortalized MO3.13 human oligodendrocyte cell line as a model.

2.3 Material and Methods

2.3.1 Material

2.3.1.1 Polyene Macrolide Antibiotics

Amphotericin Methyl Ester (AME) and Amphotericin B (AMB) (see section 1.4.3.2 Material and Methods)

Vehicle (Negative Control) Preparation (see section 1.4.3.2 Material and Methods)

Antibiotic Reconstitution (see section 1.4.3.2 Material and Methods)

Reconstitutions for Cell Culture: The final dilution concentration of AME/AMB (0.5, 1.0, 5, 10, 20 and 30 µg/ml) were made fresh from the stock using 5% dextrose and further diluted with serum media to reach the final volume.

Sterilization (see section 1.4.3.2 Material and Methods)

2.3.1.2 Positive Control Preparation

Apoptosis: Staurosporine, a potent cell-permeable inhibitor of protein kinase C that induces apoptosis (Bertrand et al., 1994; Craighead et al., 1999), was purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was ready-made at a concentration of 100 µl/214 ml (1 mM). One µM working solution was prepared from the stock for incubation with cells.

2.3.2 Methods

2.3.2.1 Extracellular Matrix

Glass cover slips (22-mm x 22-mm) were coated with natural mouse laminin (Invitrogen Corp, Carlsbad, CA) prior to cells being plated. Natural mouse laminin (Timpl et al., 1979), isolated from the Engelbreth-Holm-Swarm (EHS) Sarcoma is the major glycoprotein of the basement membrane and serves as a substrate that enhances cell growth, motility and attachment (Mecurio, 1990). Laminin stock (1 mg/ml) was diluted according to the manufacturer's protocol for a final concentration of 20µg/ml by adding 200 µl to 10 ml of sterile ddH_20. Five hundred µl of the dilute laminin was applied on top of sterile glass cover slips placed in 35 mm cultures dish. The culture dish was incubated at 37 ^oC for one hour in a humidified incubator with 5% CO_2, after which the laminin-coated cover slips/slides were allowed to dry in the hood for a minimum of 30 minutes following aspiration of excess laminin off the cover slips. To enhance

sterility, the dry laminin coated slides were exposed to ultraviolet light for at least an hour before use.

2.3.2.2 MO3.13 Human cell line Model for Assessment of AME/AMB Neurotoxicity

Available data from Buntnix and coworkers (2003) indicate that the MO3.13 cells provide an immortalized clonal model system that can be serially passaged, and has been evaluated as a useful model employed in neurotoxicity studies (Mclaurin et al., 1995; Craighead et al., 1999). The MO3.13 cell line has been used successfully in several other studies (2000; Issa et al., 2003; Li et al., 2002; Haq et al., 2003) as a model system offering the advantage of fast proliferation, convenience of growth and laboratory manipulation in a homogenous cell population.

The human oligodendrocyte MO3.13 cell line (Mclaurin et al., 1995) is an immortalized human-human hybrid cell line expressing phenotypic characteristics of primary oligodendrocytes, created by fusing a 6-thioguanine-resistant mutant of the human rhabdomyosarcoma, a skeletal muscle tumor, with adult human oligodendrocytes via a lecithin-enhanced polyethylene glycol procedure (Craighead et al., 1999). MO3.13 cells readily differentiate in serum-free media and express several of the biochemical and morphological characteristics of mature primary oligodendroglia (Craighead et al., 1999; McLaurin et al., 1995; Li et al., 2002; Haq et al., 2003; Buntinx et al., 2003).

2.3.2.3 Cell Culture

MO3.13 oligodendrocyte cells (Cellutions Biosystems Inc., Ontario, Canada, Lot # 211209-P18) were cultured in accordance to the manufacturer's protocol. Cells were placed in 60 mm tissue culture plates (Falcon) with a high glucose (4500 mg/L) formulation of Dulbecco's Modified Eagles Medium (DMEM: Sigma D5796) without sodium pyruvate, supplemented with 10% heat inactivated fetal bovine serum (FBS). Upon thawing, cells were diluted 5-fold, placed in 60 mm tissue culture dish (Falcon) and incubated in a humidified 5% carbon dioxide (CO2) and 95 % air atmosphere at 37 ^{o}C. Attachment occurred rapidly, with viable cells on the culture plate within 4 to 6 hours, at which time the media with DMSO was removed and DMSO-free media added. Cells were checked daily for evidence of contamination. Growth media was renewed and cells sub-cultured every two days at approximately 80% confluence, by cell detachment using 0.5 ml of enzyme-free phosphate-buffered saline (PBS) based cell dissociation buffer (Gibcco/Invitrogen Corp., Carlsbad, CA), incubated for 5 min at 37 ^{o}C, followed by mechanical triturating using a flame polished Pasteur pipette. Cells were then plated at approximately 1 x10^{5} cells/ 60 mm culture dish. Cells at a density of 170,000 cells/well (approximately) were seeded in 24 well plates and laminin coated glass coverslips (placed in 35 mm culture dishes). Furthermore 42,500 cells/well were seeded in 96-well micro titer plates. To initiate differentiation, naive MO3.13 cells were grown on 60 mm culture dish as described above. Cells were then subjected to differentiation medium (DMEM with 1% FBS) 24 hrs after plating or at 80% confluence (Craighead et al., 1999; Haq et al., 2003). Cells were differentiated for 5 days and the media refreshed every 2

days. All cells were grown under similar conditions. However, two duplicate wells or culture dishes were left as controls (untreated/vehicle) in each experiment. All cell cultures were drawn from identical passage numbers between passage 5 and 20.

2.3.2.4 Characterization

Morphology Human MO3.13 glial cells were cultured in SFM over 5 days. Cells were viewed daily using a Leitz Labovert inverted microscope (Ernst Leitz GmBH., Germany) optical microscope. Images were captured using an alpha-300 SLR (Sony, Japan) camera.

Immunocytochemistry Following differentiation, cells were washed in PBS, then fixed in 4% paraformaldehyde for 10 min, washed in PBS and incubated at RT with 2% triton X-100 in PBS for 15 min. Next, cells were washed in PBS, incubated with 10 mg/ml sodium borohydrate in PBS for 10 min followed by PBS wash, then blocked for an hour with goat serum at RT. Following a PBS wash, cells were incubated with avidin solution for 15 min, washed in PBS, incubated with biotin solution for 15 min and washed in PBS. Primary incubation (1:100) was carried out with anti–rabbit myelin proteolipid (PLP) antibody (Abcam, Cambridge, MA) for 1 hr at RT followed by PBS wash. Subsequently, secondary incubation (1:200) with goat anti–rabbit IgG antibody (Abcam, Cambridge, MA) was performed for 30 min at RT followed and by PBS wash. For fluorescence analysis, cells then were incubated at RT with streptavidin conjugated to FITC and counterstained with Hoescht 33342 (Immunochemistry Technologies) for 1hr in the dark, washed in PBS and mounted on glass slides with fluoromount G (Southern Biotech,

Birmingham). The mounted coverslips were viewed using a Zeiss microscope, while a ProgRes® digital camera was used for phase-contrast and fluorescent image capture. All fluorescence procedures were carried out in the dark to limit photo bleaching of images. For analysis, 6 random fields were counted per slide and a minimum of 300 cells counted/slide.

2.3.2.5 Trypan Blue Exclusion Assay

Cells were stained with trypan blue. Briefly, differentiated MO3.13 cells seeded in 96-well microplates were incubated with serum free medium (SFM) containing appropriate concentrations of either AMB or AME: 0.5 µg, 1, 5 µg, 10 µg, 20 µg and 30 µg . A final concentration of 0.2% sterile trypan blue (Gibcco/Invitrogen Corp., Carlsbad, CA) prepared from 0.4% stock was incubated with the monolayer of adherent cells for 5 min at 37 ^{o}C. To quantify cytotoxicity for each well, triturating disaggregated cells were counted using a levy chamber (Clay Adam, New York) under a Leitz labovert inverted microscope fitted with an alpha-300 SLR (Sony, Japan) camera. Cell injury was estimated as the percentage of trypan blue staining cells/ total number of cells counted.

2.3.2.6 Propidium Iodide (PI)/ Fluorescein Diacetate (FDA) Assay

Cells plated on 22 x 22 mm glass coverslips coated with laminin were washed with PBS. While in the dark, 1µl of 2-mg/ml PI stock was mixed with 3µl of 5mg/ml FDA (sigma) stock as a double stain. The cocktail was added to 1 ml of fresh pre-warmed SFM and incubated with the cells for 30 min at 37 ^{o}C. Cells were washed with PBS and coverslips mounted for fluorescence microscopy with a Zeiss microscope equipped with CoolSnapPro camera. Slides not being assessed immediately were kept on ice or in 4 ^{o}C to limit the loss of FDA fluorescence. For analysis, 6-random fields were counted per slide. The count of PI^{+} cells and FDA^{+} cells as a percentage of total number of cells was performed manually.

2.3.2.7 Nuclear Area Factor (NAF) Evaluation

Images of nuclear phenotype changes of MO.13 cells were digitally profiled to determine the relationship of the computed NAF values to severity of injury. Stored images for each desired experiment (microscopic slides) were opened using the ImagePro 7.0 software. Utilizing the measure tool of the count size function and selecting for roundness and area simultaneously, both the nuclear area and roundness were scored for each Hoechst 33342-positive cell selected in each image of six randomly chosen fields per experiment. The data output was transferred to an excel spreadsheet where the nuclear area factor (NAF) for each cell/nuclei was computed as the product of the nuclear area and roundness (roundness*area). The NAF for each treatment group was expressed as the average +/- SEM

2.3.3 Statistics

Data from the *in vitro* studies were subjected to parametric statistical analysis using completely randomized statistical one-way analysis of variance (ANOVA) with Levene's test for unequal variances, using Medcalc 12.3.0 software (Mariakerke, Belgium).The students-Newman-Keuls (SNK) test for pair wise comparison (post-hoc analysis) was used to determine the difference between mean values. The probability values (*p*) required to attain statistical significance for all tests, from appropriate negative controls, was set at $P \leq .05$.

2.4 Results

2.4.1 Differentiated Human MO3.13 Oligodendrocyte Express Phenotypic Traits of Mature Primary Oligodendrocytes

We validated the human MO3.13 oligodendrocyte as an appropriate model for our cell culture studies. We assessed microscopically the morphological transition from Day one (D1) through Day five (D5) for the group subjected to differentiation media and compared them to the undifferentiated cells. The serum-deprived cells acquired a flattened, triangular outline with several greatly elongated cellular processes (Figure 2.1) and a prominent nucleus and nucleoli, an appearance morphologically similar to mature

primary oligodendrocytes. This contrasts the undifferentiated cells, which have short bipolar processes and an indistinguishable nuclear outline.

Differentiated MO3.13 cells demonstrated immunoreactivity for the markers of mature primary oligodendrocytes proteolipid protein (Figure 2.2; 2.3). Our data is consistent with that of others (Buntnix et al., 2003; Craighead et al., 1999; 2000, Issa et al., 2003; Li et al., 2002; Haq et al., 2003) that MO3.13 immortalized human-human hybrid cell line serve as appropriate experimental model for primary oligodendrocyte culture demonstrating comparable phenotypic markers of differentiation.

2.4.2 AMB Induces Cytotoxicity in Oligodendrocytes

In order to determine the extent of potential toxicity to the central myelin producing oligodendrocytes and the appropriate AMB and AME concentrations to employ, a quantitative dose-response profile of their cytotoxicity was carried out using dye exclusion methods (trypan blue). Cells in serum-free media for 24 hr were maintained in the presence of AMB and AME at the indicated concentrations of 0.5–30 µg/ml and analyzed at 2, 4, 6 8 and 24 hr time points. Bright field microscopic analysis revealed the lowest concentration of AMB to induce significant ($p < 0.05$ vs. control) cytotoxicity was 1µg/ml after 2 hr exposure (Figure 2.4A). Greater than 90% cell death was attained with 30 µg/ml of AMB at 24 hr. MO3.13 cells were less vulnerable to AME than AMB, with 10 µg/ml of AME (LC_{10}), being the lowest dose to induce significant

Figure 2.1 Human MO3.13 Oligodendroglia cells differentiate on withdrawal of serum

(Panel a-e) Human MO3.13 glial cells cultured without serum demonstrates morphological transformation from naïve to adult oligodendrocytes reminiscent of mature primary oligodendrocytes. They transform from bipolar cells (red arrows) with less complex processes at Day 1 (a) to multi-polar cells (blue arrows) with greater ramification of their elongated process at Day 5 (e). (Panel f-j) In contrast, the group incubated with growth media (DMEM) containing about 10% serum show very little change in bipolar morphology from Day 1 to Day 5 (red arrows). The images are representative of light micrographic analysis of live MO3.13 cell maturation. Magnification is x40.

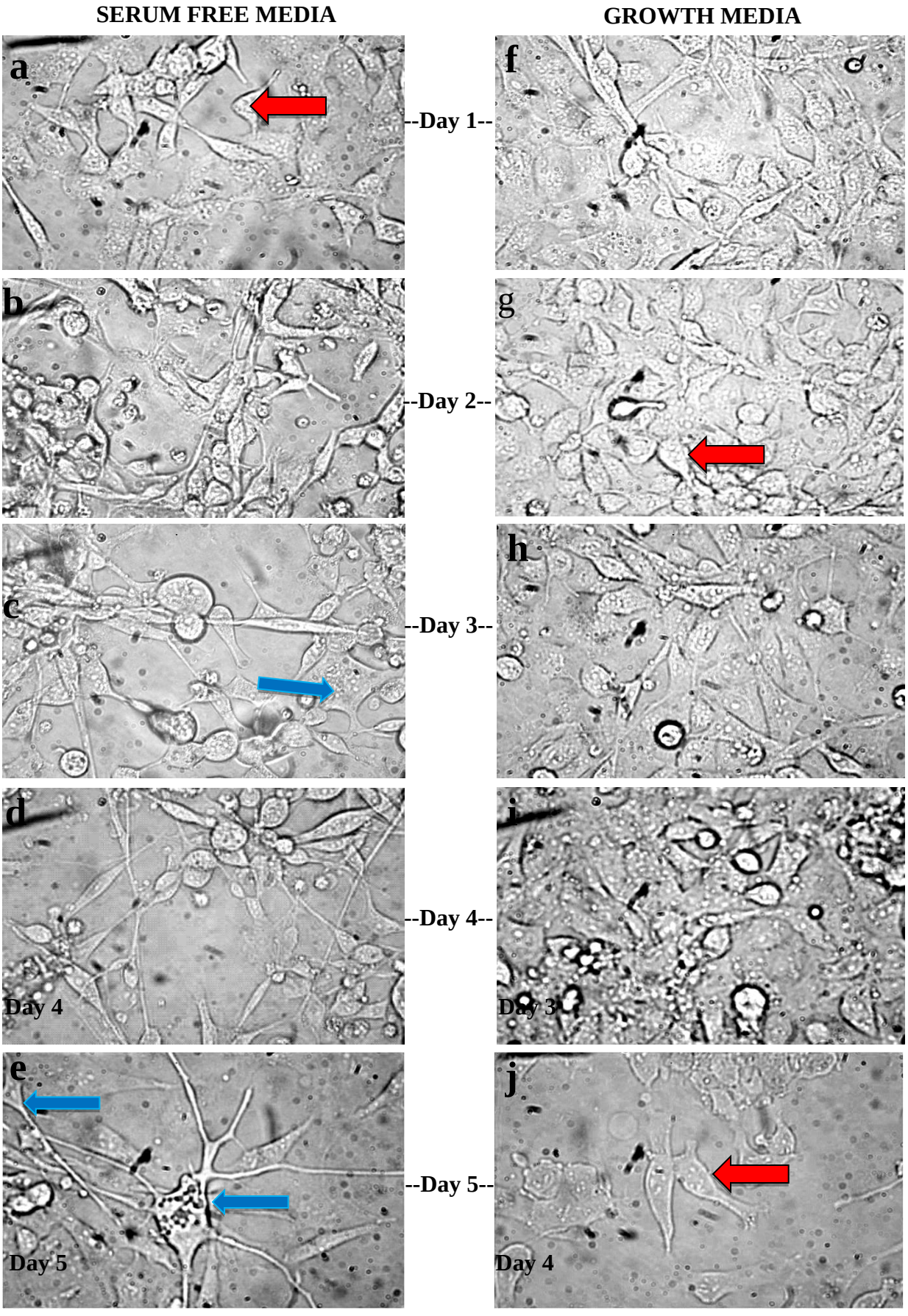

SERUM FREE MEDIA
GROWTH MEDIA
a
f
--Day 1--
b
g
--Day 2--
c
h
--Day 3--
d
i
--Day 4--
Day 4
Day 3
e
j
--Day 5--
Day 5
Day 4

Figure 2. 2 Morphology of differentiated human MO3.13 oligodendrocyte

(A) Phase contrast micrograph of MO3.13 human oligodendrocyte cell line after five days of differentiation. (B) Phase contrast micrograph representation of the differentiated cells showing a flattened, triangular phenotype (block arrows) with several elongated cellular processes indicating maturity. (C) In addition, a corresponding proteolipid protein (PLP) antibody reactivity (green) spanning both the nucleus and cytoplasm further demonstrates maturity. (D) Merged phase and immunofluorescence micrographs (B and C).

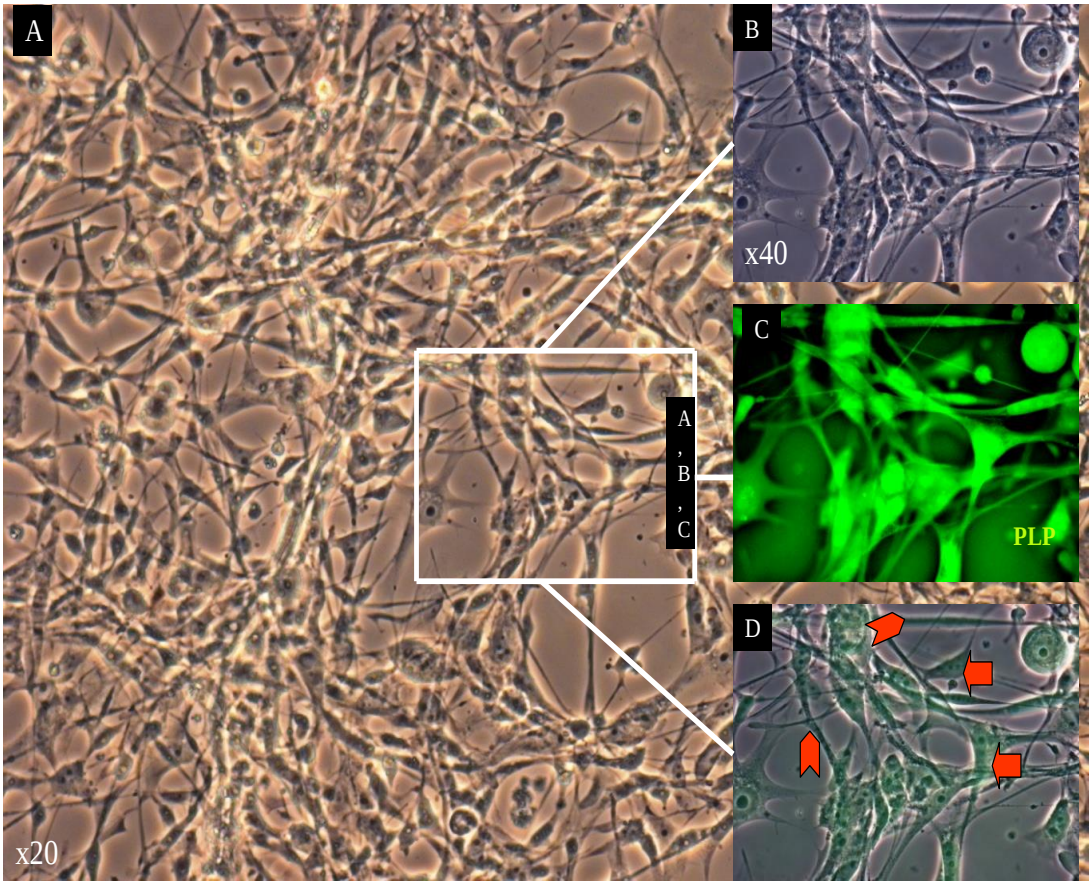
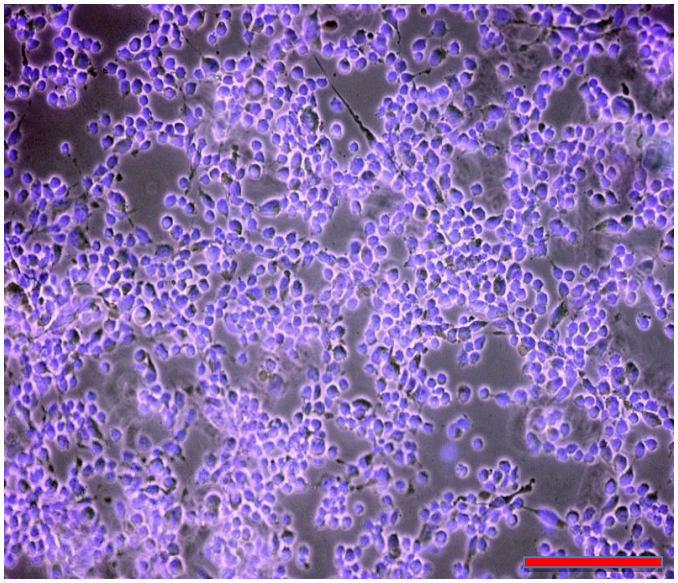


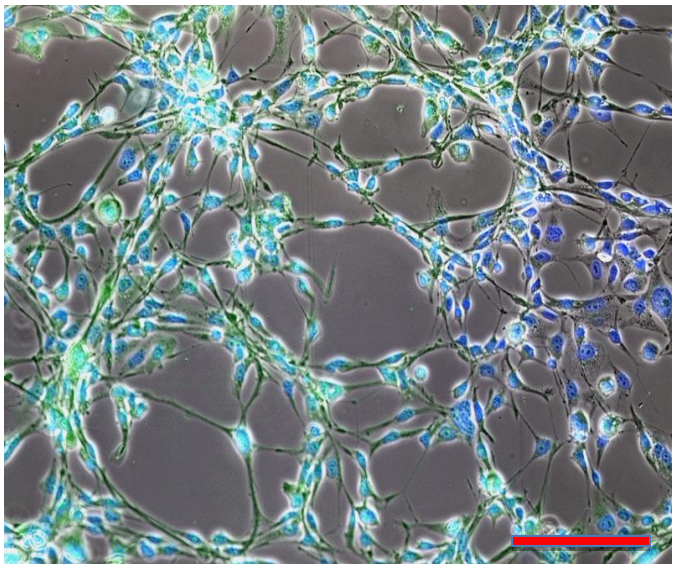
Figure 2. 3 Figure 2.3 Differentiation is almost universal at Day 5

Below are representative composite micrographs of phase contrast images and dual fluorescent labels of MO3.13 oligodendrocytes. D0 depicts undifferentiated cells 6 hours after plating. (D5) cells at Day 5 post differentiation display increased PLP (green) reactivity. Counter-stain with Hoechst nuclear stain (blue) demonstrates areas of less differentiation, depicted by low green fluorescence (PLP) and high blue fluorescence (Hoechst). Scale bar is 100 µm

D0



D5



permeability at the 2 hr time point while the highest concentration, 30µg/ml tested induced < 35 % cell death after 24 hr (Figure 2.4B).

2.4.3 AMB Induces Oligodendrocyte Cell Death

To further explore the nature of toxicity of AMB and AME in terms of oligodendrocyte cell viability, we employed epi-fluorescence techniques using dye exclusion of a DNA fluorogenic dye (PI). MO3.13 cells were exposed to increasing concentrations of AMB and AME for 2 hrs. Exposure of MO3.13 oligodendrocytes to 1µg/ml of AMB caused increases in PI staining compared to controls, indicating cytotoxicity (Figure 2.5). Similar low concentrations of AME did not cause notable PI uptake.

2.4.4 AMB Induces Vacuolations in Oligodendrocytes and Mixtures of AME and AMB Leads to Increased Toxicity

We then determined potential morphological changes induced in oligodendrocytes by the test antibiotics AMB and AME across various concentrations after 2 hrs exposures. AMB at 1 µg/ml caused moderate vacuolation or membrane blebbing in unstained live-oligodendrocytes, indicating injury to the cells (Figure 2.6). AMB treated cells at 10 µg/ml caused severe vacuolations and some cells retracted their processes, rounding up before detaching from the surface of the culture dish or coated glass coverslip.

Figure 2. 4a Cytotoxic effects of AMB

(A) Represent the percentage of dead MO3.13 cells as depicted by trypan blue-staining with exposure to 0.5-30 µg/ml concentrations AMB, and includes those lost to lysis. Each data point represents mean ± SEM of 3 independent experiments (n=3, each the mean of duplicates). * Is the lowest concentration inducing membrane permeability significantly different from its respective dextrose control (p= < 0.05). The concentration at which 50 % of the cells die (IC_{50}) is 10 µg/ml at 2 hours exposure. The concentration at which 10 % of the cells die (LC_{10}) is 1 µg/ml at 2 hours exposure.

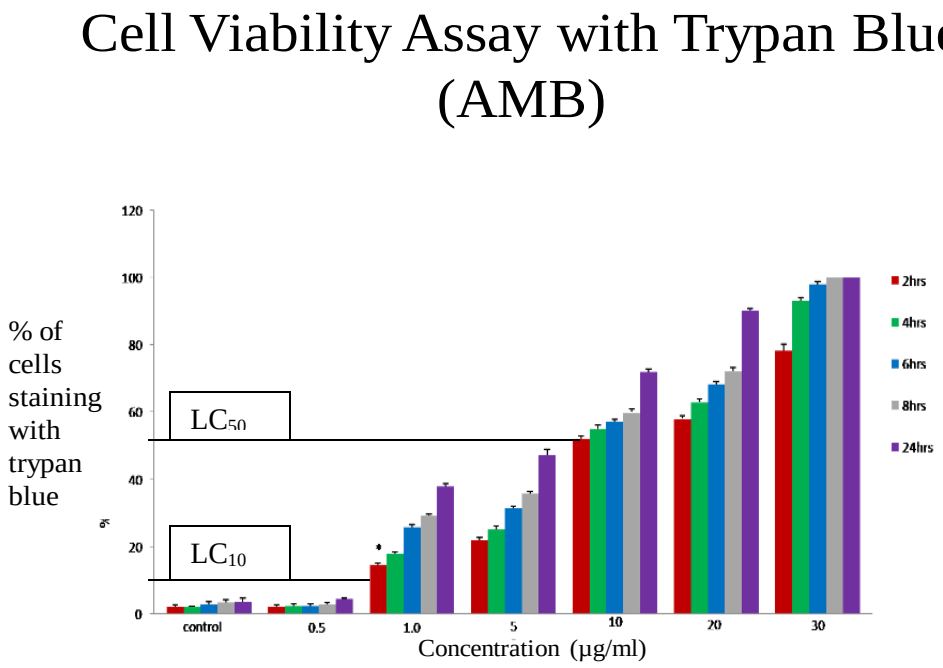
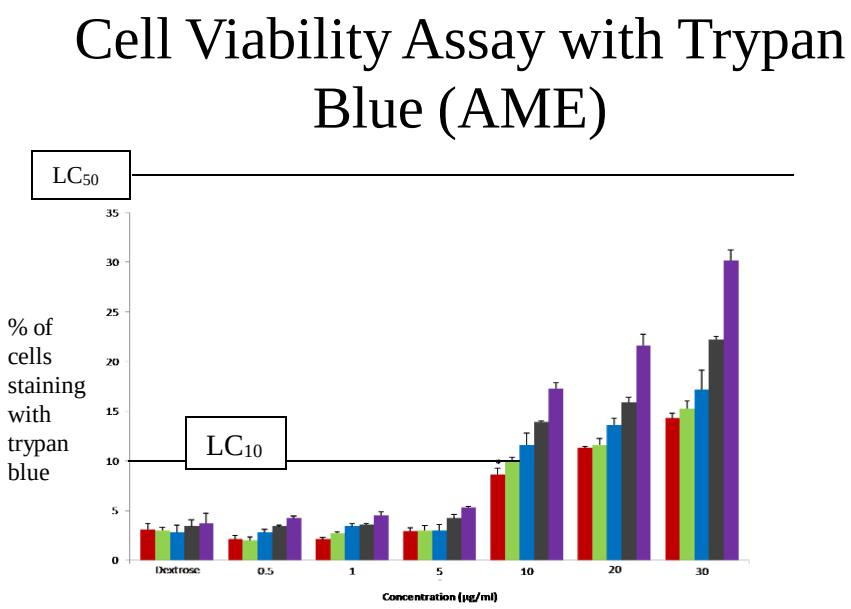


Figure 2.4b Cytotoxic effects of AME

The graph below represents AME exposed MO3.13 cells treated similarly as AMB above. The concentration at which 50 % of the cells die or lethal concentration 50 (LC_{50}) was not attained with the concentrations used. The concentration at which 10 % of the cells die (LC_{10}) is 10 µg/ml and it is the lowest concentration that induced significant cytotoxicity vs. its respective dextrose control (p= < 0.05).



Detachment is an indication of severe injury and, in some cases cell death as evidenced by 100 % staining of all detached cells with trypan blue. Cells similarly treated with 1 µg/ml AME had no visible vacuolation and only at concentrations of 10 µg/ml did we observe mild to moderate vacuolation (Figure 2.6). These data suggests that AMB, but not AME, induces injury in mature oligodendrocytes.

Combination of AMB and non-toxic concentrations of AME to produce 10% residual AMB in the mixtures can cause neurotoxicity (Reuhl et al., 1993). Figure 2.6 depicts the results of a two-hour exposure, mixtures of both AME/AMB at concentration ratios of 9:1. The final concentration for treatment was 10 µg/ml (AME = 9 µg/ml and AMB = 1 µg/ml). Considerable cytotoxicity, depicted by fragmentation of cells, was observed in the mixture group. This fragmentation contrasted the cytoxicity (retraction of processes and rounding up) observed in the AMB (1 and 10 µg/ml), AME (10 µg/ml) cells and TET positive controls. Although subjective, the cytotoxicity in the mixture group appeared to be more severe than the AMB (1 µg/ml) and AME (10 µg/ml) cells but less than the AMB (10 µg/ml) cells.

2.4.5 Low AMB Exposure Reduces MO3.13 Cell Viability

The condensation of chromatin, its arrangement at the margin of the nucleus and nuclear disintegration and cytoplasmic blebbing/fragmentation are morphological features particular to cell death induced by apoptosis (Wyllie, 1980; Roy et al., 2008)..

Figure 2. 5 Propidium Iodide assay of AMB and AME induced cell death

Acute toxicity is demonstrated by a change in plasma membrane permeability to propidium iodide (PI) after a 2 hour exposure of cells to AMB (1 and 10 µg/ml). It was only at 10 µg/ml that AME exhibited toxicity. Viable cells do to take up the red PI dye.

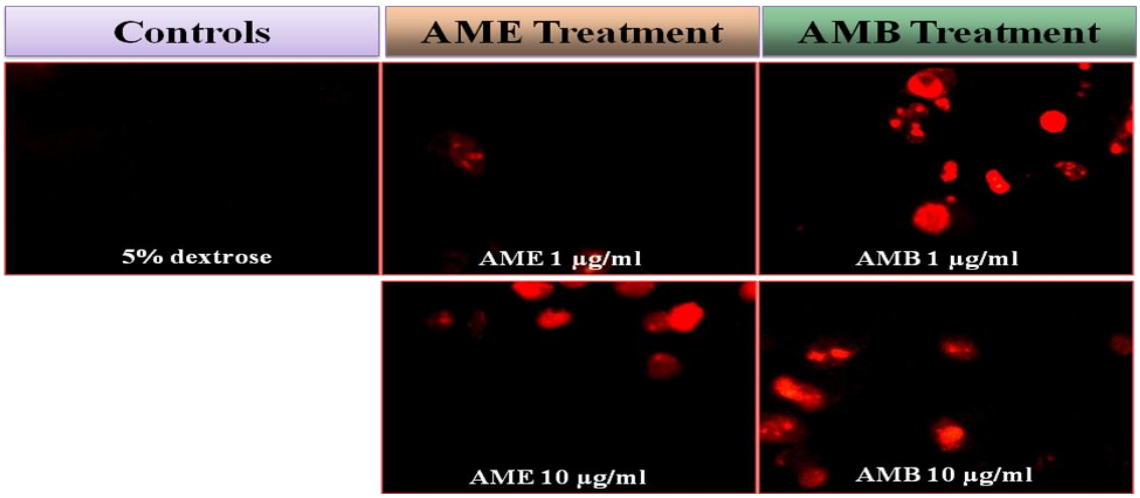
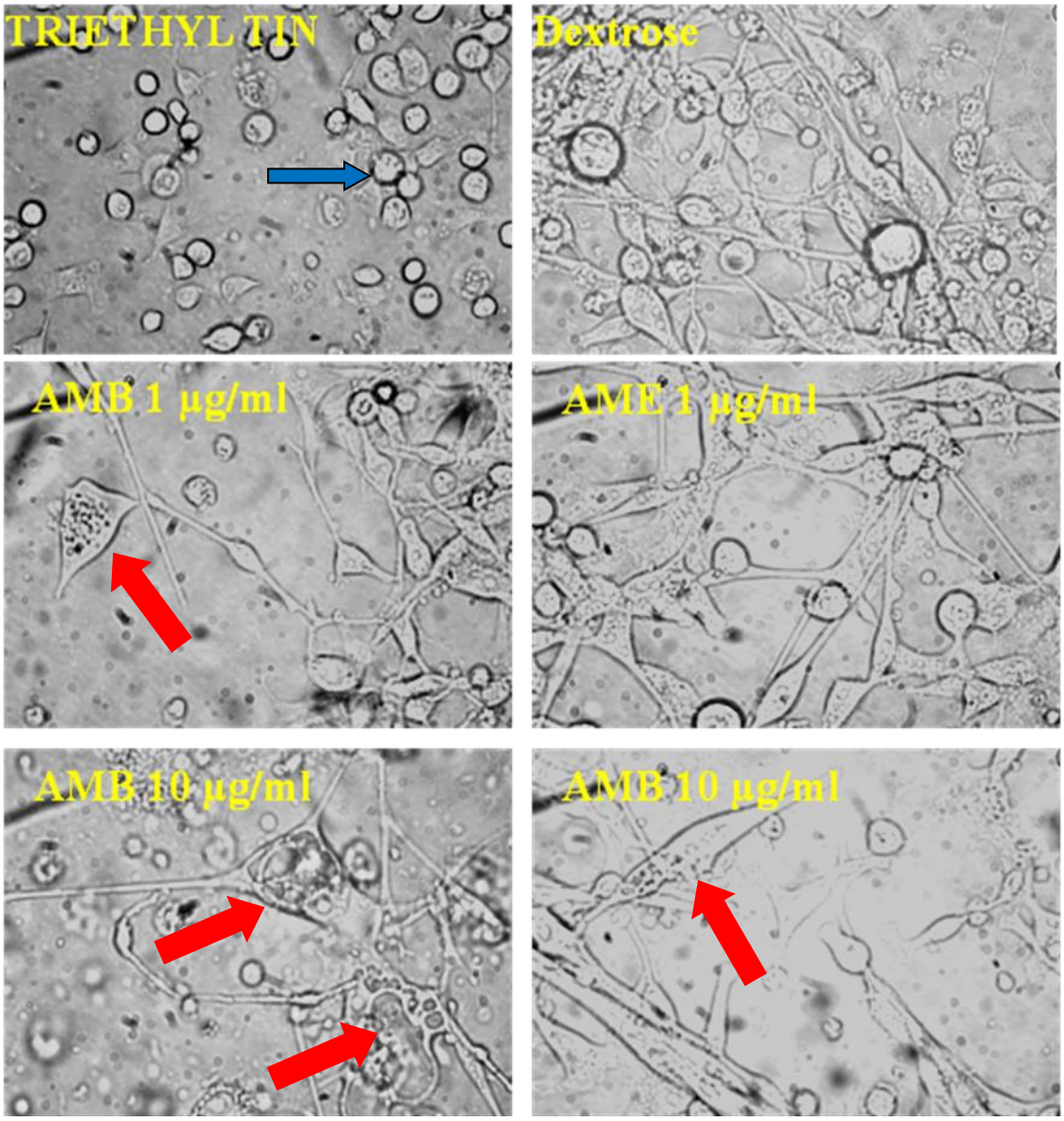
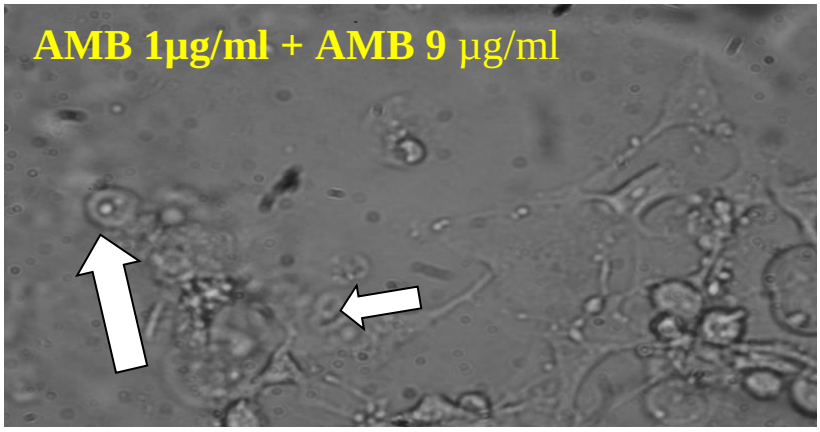


Figure 2. 6 Low concentrations of AMB alters the morphology of oligodendrocytes

Bright field image analysis of unfixed oligodendrocytes shows triethyl tin (TET) treated cells (positive control) with rounded morphology floating in media after detachment, indicative of cell death. AMB (1 µg/ml) causes rounded up and floating of ells (blue block arrows) similar to those seen in the positive control group. At 10 µg/ml of AMB, we observed cytoplasmic vacuolation with cell swelling in some of the cells (red block arrows). In contrast, changes in AME 10 µg/ml appeared subtler than that in both AMB groups. Mixtures of 9 µg/ml AME and AMB (1 µg/ml) appeared to cause more cytoxicity than AMB (1 µg/ml), manifesting more as fragmentation of the live cells than vacuolations.


TRIETHYL TIN
Dextrose
AMB 1 µg/ml
AME 1 µg/ml
AMB 10 µg/ml
AMB 10 µg/ml


AMB 1µg/ml + AMB 9 µg/ml

FDA/PI labeling (Figure 2.7) was employed to examination the role of apoptosis in AMB and AME injury; epifluorescence microscopy utilizing a dual-band filter was used to co-localize the PI and FDA dyes in the MO3.13 cells. Figure 2.7 shows changes consistent with apoptosis at low concentrations of AMB (1µg/ml) but not AME, suggesting that AMB may cause injury to oligodendrocytes by an apoptotic mechanism at low doses

2.4.6 Assessment of Nuclear Morphology

Shrunken nuclei display hyperchromasia, leading to marked fluorescence when labeled with cell permeant nuclear dyes such as Hoechst 33342 (Buntinix et al, 2004). In this study a similar pattern of fluorescent nuclear condensation, marginalization and fragmentation of MO3.13 cells on microscopy is observed with low AMB (1 µg/ml) and high AME (10 µg/ml) concentrations after 2 hrs of incubation. This was comparable to the staurosporine (positive controls) treated cells (Figure 2.8 AB), in agreement with our earlier findings using PI dye (see Figure 2.7). Untreated dextrose control cells lacked these phenotypic traits as did with cells treated with 1 µg/ml of AME. These data also reinforce the observation that MO3.13 cells may be vulnerable at low AMB via an apoptotic pattern of death cell death.

Employing computerized morphometric techniques to reduce observer-dependent bias and increase sensitivity in scoring, the phenotypic characteristics of M03.13 cells were further studied after labeling with Hoechst 33342 nuclear dye. This was the best method to verify whether disparities in chromatin condensation and nuclear size are present after experimental treatment with AMB and AME. The nuclear area factor was

Figure 2. 7 Qualitative assay of MO3.13 cell viability following AMB exposure

Green fluorescence indicates viable cells, which retain the vital dye fluorescein diacetate (FDA) and are demonstrated by green arrows in a majority of cells in the control group and cells treated with 1µg/ml AME. In contrast, the yellow to orange fluorescence indicates cells membranes permeable to propidium iodide and FDA (PI+/FDA+ cells) indicating early phase of death (orange block arrows) and are observed mostly in AMB (1µg/ml) treated cells and to a lesser extent in 10µg/ml AME treated cells. The yellow block arrows depict condensed chromatin and fragmented nuclei (apoptotic nuclei). PI-positive nuclei (red) shown by red block arrows may indicate late stage apoptosis or necrosis seen chiefly in cells incubated with10µg/ml AMB. To increase the validity of these assay to detect apoptosis we employed molecular probes in the next chapter. Magnification is x40

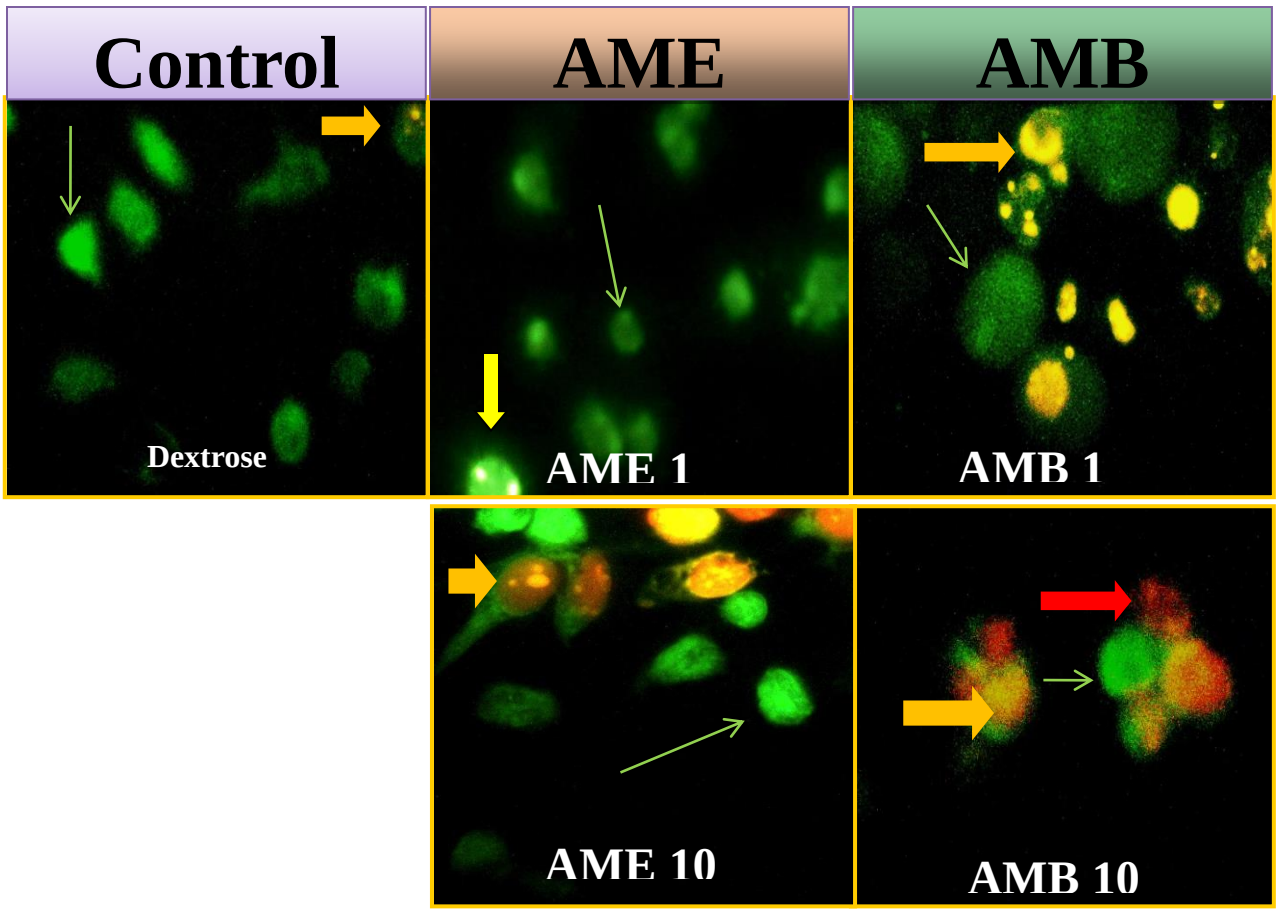

Control
AME
AMB
Dextrose
AME 1
AMB 1
AME 10
AMB 10

Figure 2. 8 Low Concentrations of AMB causes an apoptotic phenotype in glial cells (A and B) The known apoptosis inducer staurosporin provokes nuclear morphological alterations (arrows) of condensation/margination and fragmentation similar to (C and D) AMB (1µg/ml) treated cells. (E and F), although at10 µg/ml AMB appears to cause more cytotoxicity, cells with apoptotic characteristics were more common than in the lower concentration group of AMB. While there was no obvious difference in the morphology of (I and J) the controls and (G, H), the 1µg/ml AME group, the (K and L) 10 µg/ml AME exposed cells appear to have changes similar (to a lesser extent) to the low concentration AMB group. Scale bar is 100 µm.

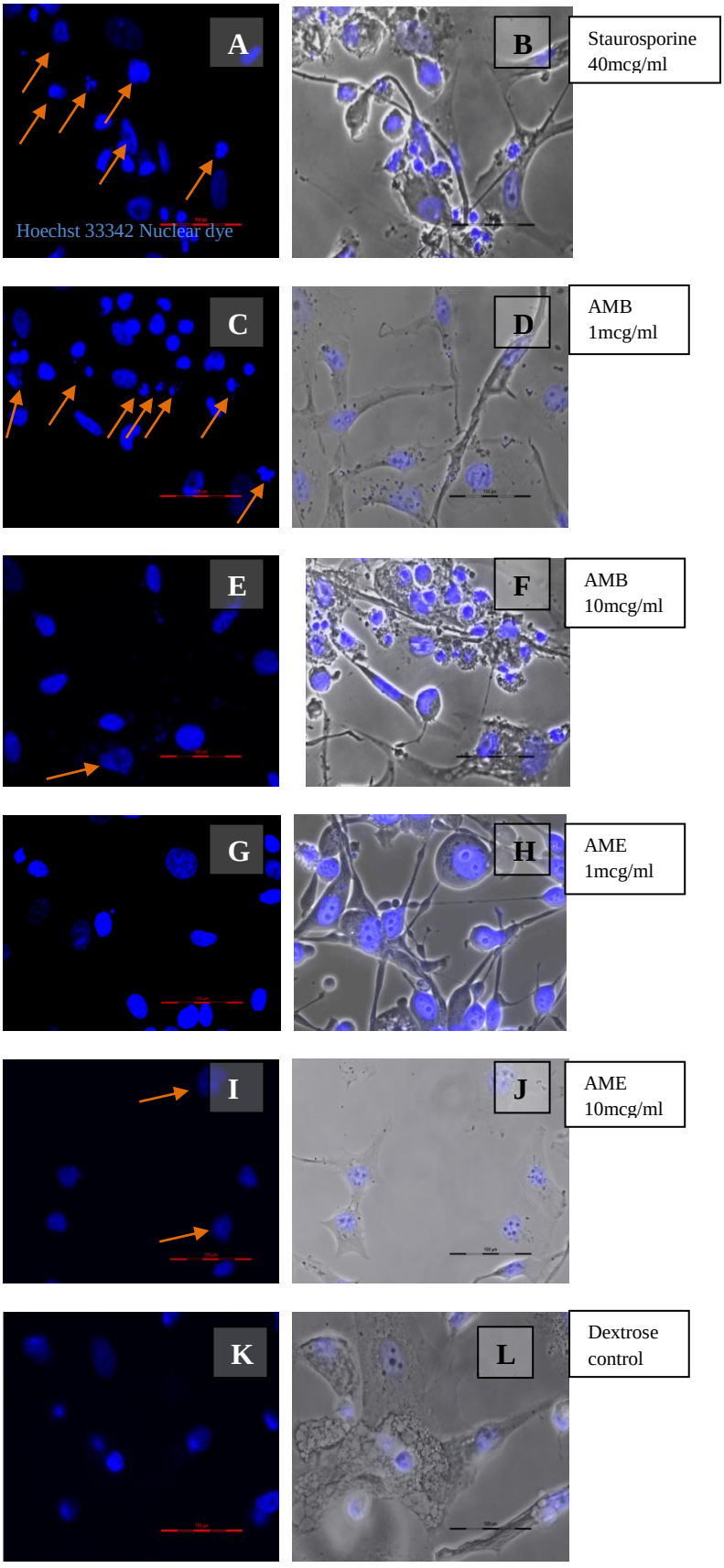
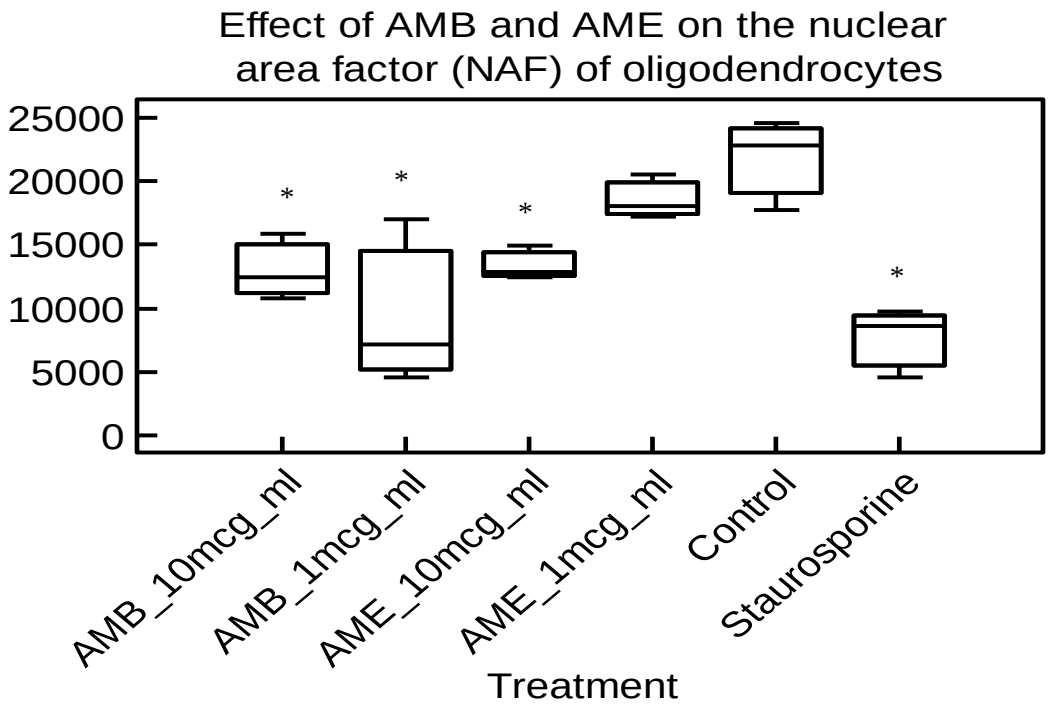

A
Hoechst 33342 Nuclear dye
B
Staurosporine
40mcg/ml
C
D
AMB
1mcg/ml
E
F
AMB
10mcg/ml
G
H
AME
1mcg/ml
I
J
AME
10mcg/ml
K
L
Dextrose
control

Figure 2. 9 Quantification of apoptotic nuclei

For nuclear morphometric analysis of AMB and AME treated cells including the vehicle controls and the positive control (Staurosporine), six random fields per slide were chosen and the area and roundness were scored for Hoechst 33342-positive cells. The nuclear area factor (NAF) for each nucleus was computed as the product of the area and roundness (roundness*area) (Lieven et al., 2003). The NAF for each treatment group was expressed as the average +/- SEM of 3-independent experiment (n=3, each the mean of duplicates). *p= < 0.05 compared to vehicle control.



examined after 2 hours incubation with AMB and AME (Figure 2.9). Significant differences between AMB (1 µg/ml) and dextrose controls ($p < .05$) were noted (Figure 2.9). Similarly, a significant difference in NAF ($p < .05$) was recorded for high concentrations of AME when compared to controls.

2.5 Discussion

The studies in this chapter examined the potential *in vitro* cytotoxicities of the commonly used polyene macrolide antifungal amphotericin B and its more soluble analog AME in differentiated MO3.13 human oligodendrocyte cells. Polyenes are known to bind sterols in membranes causing membrane pore formation that leads to loss of cellular electrolytes and consequent cell demise that is more in fungal than host cells (Schaffner and Mechlinski, 1972). The major finding of this study was to document and characterize the cell membrane perturbation by AMB and AME. Our results show that AMB exhibited considerable cytotoxicity when compared to AME. In addition, we were able to show for the first time that AMB is lethal to oligodendrocytes at low concentration of 1µg/ml by inducing membrane permeability and consequent apoptosis *in vitro*. We further demonstrated that pure AME is not toxic to oligodendrocytes at low concentrations of 1µg/ml. This study also suggests that mixtures of AME and AMB can be highly toxic to oligodendrocytes.

2.5.1 Phenotypic Expression of Adult Primary Oligodendrocytes Features in Differentiated MO3.13 Glia Cells

We first confirmed that differentiated MO3.13 oligodendrocytes expressed the phenotypic characteristics of mature primary oligodendrocytes. The appearance of extensive PLP-positive cells at day five in human MO3.13 oligodendroglia cell culture after exposure to serum-free growth media (Figure 2.2;2.3) is consistent with the biochemical and phenotypic changes observed in primary oligodendrocytes and previous reports of MO3.13 cells cultured without serum (Haq et al., 2003; Giri et al., 2008). The protein PLP is among the first myelin proteins to be expressed in measurable quantity (Baumann and Pham-Dinh, 2001) in oligodendrocytes and is indicative of maturation of oligodendrocyte precursor cells (Haq et al., 2003). Previous studies have suggested that the withdrawal of serum leads to decrease in cyclin and cyclin-dependent kinases in the cell, subsequently inducing differentiation (Crowe et al., 1998).

In addition to expressing mature myelin proteins such as PLP, our studies show that the MO3.13 cells assumed morphological changes reminiscent of mature primary oligodendrocytes. The mature MO3.13 cells demonstrated a flattened and often triangular morphology with characteristically elongated processes that may allow for ease of axonal myelination, as well characteristic large nuclei with prominent nucleoli (Figure 2.1; 2.2).

2.5.2 AMB is More Cytotoxic than AME

MO3.13 oligodendrocytes were vulnerable to treatment with AMB at low concentrations but not to AME. This study provided evidence of trypan blue uptake into the cytosol (Figure 2.4), indicative of cell membrane compromise and toxicity in 1 µg/ml AMB group that was consistent with previous studies (Racis et al., 1990). Conversely, similar concentrations of AME failed to induce cytotoxicity. However, AME at high concentrations (10 µg/ml) induced significant cytotoxicity after 2 hrs exposure. An equivalent concentration of AMB was significantly more toxic ($p< 0.05$). This observation is consistent with the *in vitro* work of Racis and colleagues (1990) that first reported the direct cytotoxic effect of AMB being ten-times more potent than pure formulations of AME. Several *in vitro* studies using non-neural cells provide further support that AMB is more toxic than AME (Fischer et al., 1975; 1976; Bonner et al., 1976). The reason for AMB's higher toxicity may be due AME's better aqueous solubility that confers a lower toxicity than AMB. AMB's ability to form intrinsic intra-molecular self-aggregates, in stark contrast to AME is related to its insolubility and higher toxicity (Mazerski et al., 1990; Bratjburg and Bolard, 1996).

2.5.3 Low Concentrations of AME Do Not Induce Oligodendrocyte Cytotoxicity

Our original hypothesis was that pure AME is not toxic to oligodendrocytes while AMB is cytotoxic. However, it should be noted that significant amounts of AMB (but not AME) are bound to tissues and plasma proteins, making it difficult to predict how much AMB enters the cell. Thus, reports of serum levels of AMB may actually overestimate

how much finally reaches cells, as the assumption is 100% entry. With this in mind, we carried out the current studies, ensuring the media had negligible serum proteins.

Although both AMB and AME are known cell membrane active compounds that induce pore formation with subsequent loss of intracellular ions (Lampen, 1969), we did not observe cytotoxicity as indexed by PI uptake with low concentrations of AME (1 µg/ml) in MO3.13 cells. At high doses of 10 µg/ml, cytotoxicity in oligodendrocytes appeared (Figure 2.6; Figure 2.7) with AME. AMB at 1 µg/ml concentrations caused cytotoxicity as evidenced by PI uptake that was about a ten times higher than AME, reflecting the difference in their LC_{50}. The reasons for AMB's higher toxicity may be connected to its high lipid solubility as previously discussed, and its greater propensity to bind lipid membranes more than AME, which is more hydrophilic.

2.5.4 AMB Induces Vacuolations and Mixtures of AME and AMB are Cytotoxic

We observed cytoplasmic vacuolation in oligodendrocytes treated with AMB. This is consistent with previous accounts that vacuolation may result in some oligodendrocyte dysfunction that is related to myelin sheath disruption (Watanabe et al., 1976). In this study, AMB exposure caused overt morphological changes in live unstained MO3.13 cells as seen on light microscopy. AMB (1 µg/ml) unlike a similar concentration of AME at 2 hrs post treatment reveal membrane blebbing and cell swelling. Numerous cytoplasmic vacuoles seen at higher AMB concentrations (Figure 3.5) were accompanied by displaced nuclei. This cytopathology appeared at higher

concentrations of AME but were not as severe as those in the low dose AMB groups. Membrane blebbing/vacuolations is a characteristic feature of apoptosis (Crowe et al., 1998).

Significant increases seen in oligodendrocyte toxicity with mixtures of AME contaminated AMB in this study were consistent with histopathological reports of leukoencephalopathy seen with earlier use of AME contaminated with up to 8-10 % AMB (Hoeprich et al., 1988., Reuhl et al., 1993). Our observation (Figure 2.8), although subjective, shows that up to 10% content of AMB in AME preparations has the potential to cause toxicity. Our results were variable and the nature of the vacuoles posed a challenge in identifying and quantifying the lesions. One way to improve this experiment may be to label the membranes of the live cells with fluorescein-tagged phalloidine and subsequently determine the image intensity or morphometric alterations using automated image software such as media cybernetics ImagePro 7.0 as parameters to measure the injury. Caution should be exercised when comparing this *in vitro* result with i.v administration in animals or humans since the cell culture studies bypass the pharmacokinetic constraints that determine tissue levels *in vivo*.

2.5.5 Low AME Concentrations Do Not Induce Cell Death in Oligodendrocytes

Our current study provides evidence that low concentrations of AMB (1 µg/ml) reduce oligodendrocyte viability as indexed by PI uptake and loss of FDA green fluorescence. In contrast, AME (1 µg/ml) did not reduce cell viability (Figure 2.7). The

staining pattern was important to distinguishing the modality of cell injury or lethality. In Figure 2.7, the PI-stained nuclei (with condensation and fragmentation) in FDA positive cells (indicating functional esterases) reflect the requirement of ATP as indicated by functional esterase that catalyzes FDA fluorescence. This pattern, observed in the 1 µg/ml AMB group is consistent with apoptotic change. Our results show that at higher concentrations (10 µg/ml) apoptotic change in AME-cells is evident whereas the AMB – cells appear to undergo necrosis.

The loss of FDA fluorescence indicates a decreased ATP (loss of esterases) with attendant increase in cell membrane permeability and increased PI fluorescence. The reason for this dichotomy (necrosis and apoptosis) is unclear. It may imply that low AMB concentrations cause membrane perturbation and cell injury (known mechanism) even when the cell is viable (FDA positive) and at higher concentrations causes necrotic death. Alternately, the FDA- PI+ cells in the high AMB concentration could represent the end stages of programmed cell death. Nevertheless, accruing data provides experimental evidence that AMB can cause cells to dies by necrosis or apoptosis (Selamet et al., 1999; Marklund et al., 2001; Phillips et al., 2003; Odabasi et al., 2009; Al-dhaheri and Douglas 2010; Varlam et al., 2010). Apoptotic change has also been suggested as a mechanism behind the reversibility of some of AMB's side effects (Varlam et al., 2010). However, data examining apoptosis as a mechanism of AME toxicity is lacking. To further characterize the apoptotic change we employed semi-automated techniques using cell permeant DNA dyes.

2.5.6 Apoptotic Nuclear Morphology is Seen at Low AMB Concentrations

Two distinct features of apoptosis are chromatin condensation and apoptotic body formation (cytoplasmic fragmentation). In this study we examined the effects of AMB and AME on the induction of apoptotic phenotype in the MO3.13 oligodendrocyte nucleus using the cell permeant nuclear stain (Hoechst 33342). Apoptotic bodies along with chromatin condensation and margination of chromatin to the periphery of the nucleus (Wylie, 1980) seen with AMB treatment in these glia cells support the growing evidence that AMB is neurotoxic (Reuhl et al., 1993; Racis et al., 1990) and can cause cells to die by apoptosis (Varlam et al., 2001). Such strong evidence is consistent with our observations that oligodendrocytes die *in vivo* by apoptosis in response to AMB exposure.

Indeed, 1 µg/ml AMB induced a significant ($p < 0.05$ vs. controls) apoptotic phenotypic change (Figure 2.8; 2.9), while similar concentrations of AME (1 µg/ml) produced insignificant nuclear effects. The reason for this difference is not completely clear; it may be due to AMB having a greater affinity for the cholesterol rich membrane of the oligodendrocyte cell membrane than AME. Although there is a paucity of experimental evidence comparing AMB/AME and their effect on CNS cholesterol and other brain lipids such as sphingomyelin, perhaps, like with non-neural lipids, AMB may bind CNS lipids more avidly than AME. One important point is that this difference in toxicity is not merely a consequence of the compounds' PB/PK, since the cell culture experiments eliminates all distribution effects.

Our study confirms that AME is less cytotoxic to oligodendrocytes than the commonly used AMB. The mode of adverse outcome with AMB may in part involve apoptosis in addition to its well-known cell membrane perturbation. The study specifies additional comparative AMB and pure AME mechanistic studies to examine the specific apoptotic pathways that may contribute to AME and AMB cytotoxicity. We next compared both drugs *in vitro* using molecular probes to further characterize the morphological and biochemical changes associated with the apoptotic nuclear phenotype observed in this chapter.

Chapter 3

3 *In Vitro* Mitochondrial Membrane Effects of AME and AMB

3.1 Abstract

This study explores the mitochondrial membrane activity of Amphotericin B (AMB) and Amphotericin B methyl ester (AME) in oligodendrocytes. Neurotoxicological investigations of the highly-toxic AMB, medicine's best defense against lethal fungal infections, demonstrate it is a myelin toxicant. The less toxic, highly efficacious water soluble analog (AME) was withdrawn from clinical use over 30 years ago following reports of neurotoxicity. Investigations in our laboratory using highly-purified AME suggest that the reported neurotoxicity likely resulted from contamination by AMB and involves oligodendrocyte apoptotic changes. To test this hypothesis, immortalized human oligodendroglial cells (MO3.13) were treated with low concentrations (1 µg/ml) and high concentrations (10 µg/ml) of AMB and AME. Caspase activity, demonstrative of apoptosis, was identified using fluorescent linked inhibition of caspases (FLICA) and NucView^{TM} 488 labeling. Low AMB concentrations induced activation of caspase-3, caspase-9 but not-8 and -10. Cytochrome *c* release and mitochondrial membrane potential ($\Delta\Psi_M$) loss with AMB treatment suggested mitochondrial involvement. Significantly diminished $\Delta\Psi_M$ with AMB ($p < .05$ vs. controls) was demonstrated by the increase in the green fluorescence of the JC-1dye (5,

5', 6, 6'-tetra chloro-1, 1', 3, 3'-teraethylbenzamidazolcarbocyanine iodide) monomers vs. the red (punctuate) fluorescence of JC-1 aggregates. Trolox, an antioxidant mitochondrial membrane pore inhibitor did not prevent $\Delta\Psi_M$ loss in cells treated with 1 µg/ml AMB ($p < .05$ vs. controls) following JC-1dye analysis. These data suggest that AMB's cytotoxicity towards mature oligodendrocytes is mediated in part by apoptosis with the mitochondria playing a central role. Highly purified AME was not toxic to oligodendrocytes.

3.2 Introduction

Invasive fungal infection remains a serious challenge in medicine, with significant mortality, especially with the disseminated meningo-cerebral variant. AMB, a polyene macrolide antibiotic, is the first-line drug for invasive fungal therapy since its clinical inception in the mid-1950s. However, its use is limited by serious side effects, a source of significant morbidity and mortality. AME (Schaffner and Mechlinski, 1972), a less toxic semi synthetic derivative of AMB, appears to be the best alternative developed so far by structural modification of the AMB structure. AME mirrors AMB's exceptional antifungal efficacy except has a better therapeutic index. Nevertheless, its clinical use was terminated due to reports of leukoencephalopathy in trials in which AME preparations were subsequently shown to be significantly contaminated with AMB (Ellis et al., 1982; Hoeprich et al., 1988). In literature, the accepted dogma on how both drugs kill fungi and possibly host cells is by induction of pores in their lipid-rich membranes.

However, complete understanding of the molecular events leading to host cell toxicity remains elusive.

It has been postulated that membrane depolarization from loss of ions such as potassium may trigger mitochondrial membrane depolarization following cell membrane perturbation by polyenes, in particular, AMB. Cell membrane permeabilization may also initiate influx of several ions for example, calcium (Cohen e al., 2010). This in turn, may initiate mitochondrial mediated apoptosis (Marklund et al., 2001). Apoptosis may contribute to mammalian kidney cells injury after exposure to AMB (Varlam et al., 2010). AMB can also induce apoptosis in fungi (Robson et al., 2004). Cells dying by apoptosis (or programmed cell death) display several characteristics such as annexin V binding of exposed of phosphatidyl serine on the outer membrane, TUNEL (terminal deoxyneuclotidyl transferase dUTP nick end labeling) assay, sensitive DNA damage, including nuclear chromatin margination and fragmentation of nuclei that are easily determined on morphometry (Phillips et al., 2003). In addition, biochemical characteristics such as caspase activation easily detectable using molecular probes and can be blocked by caspase inhibitors. Recent *In vitro* and *In vivo* work in our laboratory indicates that AMB but not AME at low concentrations can induce an apoptotic phenotype on non- neural cells/oligodendrocytes.

The mechanistic underpinning of AMB and tainted-AME leukoencephalopathy has not been characterized. Highly purified AME has a good safety profile. Its excellent water solubility, and favorable physiochemical and pharmacokinetic properties likely enhance its good toxicity profile (Mechlinski and Schaffner, 1972; Hoeprich et al., 1988). However, controversies surrounding neurotoxicity from its earlier human use (Ellis et al.,

1982; Hoeprich et al., 1988) raise important questions about its safety profile and mechanism of adverse outcomes that need to be addressed: Does highly purified AME cause neurotoxicity? Alternatively, does AMB a significant contaminant found in the earlier AME preparations cause neurotoxicity? Can either drug alter mitochondrial membrane permeability (MMP) by diminishing $\Delta\Psi_M$?

This study was designed to addresses these questions by examining AMB and AME's potential to cause MMP. Our results suggest a role for the mitochondria in AMB induced apoptotic pathways, with consequent neurotoxicity.

3.3 Materials and Methods

3.3.1 Material:

3.3.1.1 Polyene Macrolide Antibiotics (See Material and Methods in Chapter 2)

3.3.1.2 Positive Control Preparation (Apoptosis)

Mitochondrial Membrane Depolarization: A stock concentration of 50 mM carbonylcyanide m- chlorophenylhydrazone (CCCP) was purchased from Immunochemistry Technologies (Bloomington, MN) and diluted to 50 µM for treatment. CCCP is a mitochondrial uncoupling agent that diffuses freely through the mitochondrial membranes abolishing the mitochondrial membrane potential.

3.1.4 Chemoprevention Preparation

Trolox 6-hydroxy-2,57,8-tetramethylchrman-2 carboxylic acid, a mitochondrial pore transition inhibitor and an antioxidant water soluble derivative of vitamin E (Gross et al., 1999) was obtained from EMD Bioscience (La Jolla, CA) as a 500 mg vial of lyophilized powder. A 500x stock was prepared and diluted to give a treatment concentration of 100 µM.

Caspase-3 inhibitor The InSolution^{TM} caspase-3 inhibitor II (Calbiochem, La Jolla, CA) was purchased as a 250 µg/75 µl (5mM) solution. It is a general caspase inhibitor (Z-DEVD-FMK) that inhibits caspase-3 activity. Characteristically, its cell membrane permeant qualities allow for its use in live cell studies.

3.3.2 Methods

3.3.2.1 Extracellular Matrix; 3.3.1 Cell Culture Technique; 3.2.3. Treatment Design; 3.3.2.4 Characterization (See Material and Methods in Chapter 2)

3.3.2. Detection of Apoptosis

3.3.2.1 Caspase-3 Assay

Caspase-3 enzyme activity was assessed with NucView™ assay kit (Biotium Inc., Hayward, CA). Cells grown on laminin coated coverslips in a serum-free environment were loaded with NucView™ 488 dye according to the manufacturer's protocol. Briefly, in the dark, media of AMB and AME treated cells was removed after 2 hours of exposure and 1µl NucView™ 488 was added to 1 ml of fresh pre-warmed SFM. Cells were incubated for 30 min at 37 º C and counterstained with Hoechst nuclear dye. The cells were washed with PBS and coverslipped for fluorescent microscopy. The number of the caspase-3 positive cells treated and control samples were compared to determine the presence of caspase-3 activation. Images were captured using ProgRes® C14^{plus} / CapturePro 2.8.8 camera system and processed with Pro-Plus 7.0 software.

3.3.2.2 Caspase-8, 9, 10 Assays

The intrinsic (Caspase-9) and extrinsic (Caspase-8 and -10) pathways of apoptosis were examined with FLICA ™ assay kit. The FLICA™ apoptosis detection method (ImmunoChemistry Technologies, Bloomington, MN), is based on the fluorochrome inhibition of caspases (FLICA)**.** Once inside the cell, the fluorochrome binds covalently to the active caspase enzyme. Briefly, cells incubated in the presence of serum free DMEM were washed with PBS. A slight modification of the vendor's staining protocol was used for cell staining. Old medium of treated and untreated cells was removed and 10 µl of the appropriate FLICA ™ reagent was added to 1 ml of fresh pre-warmed serum free media (with 1% FBS). Cells were incubated for an hour at 37 ºC, and then washed

with PBS and coverslipped for live fluorescence microscopy. To preserve cells for future microscopy, cells were post-fixed by adding 1-2 drops of ICT's formaldehyde solution (#636) washed with PBS and mounted. In contrast to absolute ethanol or ethanol, this fixative does not interfere with the FLICA label. Images were analyzed using a Zeiss Axiophot microscope fitted with a ProgRes® C14^{plus} / CapturePro 2.8.8 camera and Image Pro-Plus 7.0 software.

3.3.3. Mitochondrial Membrane Permeability (MMP) and ΔΨ m Detection

3.3.3.1 MitoPT^{TM} JC - 1 Staining and Measurement of $\Delta\Psi_m$ in Live Cells

Cells cultured on laminin covered glass slides were loaded with the MitoPT^{TM} JC–1 dye as described by the manufacturer. Cells were maintained in serum free medium, and then washed with PBS. Cells were incubated for 15 min in a 1x concentration of JC–1 dye along with the DNA dye Hoescht 33342, washed with 1x assay buffer (ImmunoChemistry Technologies) and coverslipped for live-cell viewing and photomicrography. The cells were then preserved for storage and future assessment using a formaldehyde-based fixative. The images were acquired using a Zeiss Axiophot microscope attached to a ProgRes® C14^{plus} / CapturePro 2.8.8 camera. A minimum of 18 cells were counted per slide from a minimum of 6 random fields chosen for each slide. All ratios were computed only after the average background fluorescence values were subtracted from initial fluorescence measurements (per pixel). Alternately, we

determined that the use of the black balance feature of the camera software was an easy and reliable way to correct foe the background intensity.

3.3.3.2 MitoView™ 633 Staining for MMP Assay in Fixed Cells

Cells were stained using a slight modification of the manufacturer's protocol. Briefly, cells grown on glass coverslips were covered with 1 ml of pre-warmed SFM containing 10 µl of 0.25 mg/ml MitoView™ 633 stock. Cells were then incubated with the probe for 30 min. The loading solution was replaced with fresh medium or PBS and coverslipped for live microscopy, and subsequently then fixed with formaldehyde, and mounted for storage and future microscopy. Fixing in formaldehyde does not affect the fluorescence emission of the dye. Images were viewed using a Zeiss Axiophot microscope fitted with a Sony Alpha-300 camera.

3.3.3.3 Detection of Cytochrome *c* Release

Differentiated MO3.13 cells in SFM were washed in PBS, fixed in 4% paraformaldehyde for 10 min, washed in PBS and incubated at RT with 2% triton X-100 in PBS for 10 min. Cells then were washed in PBS and incubated with 10 mg/ml sodium borohydrate in PBS for 5 min followed by another PBS wash. Cells were blocked for 30 min with goat serum at RT and incubated (1:500) in primary antibody (anti-mouse

cytochrome *c*) (Pharmingen, San Diego, CA) for 20 min at RT followed by PBS wash. Afterward, secondary incubation (1:2000) in the dark was carried out with goat anti–mouse IgG (Alexa Fluor 568) antibody (Molecular Probes, Eugene, OR) for 20 min at room temperature. Subsequently, cells were washed in PBS and then mounted on glass slides. Epi-fluorescent and phase-contrast images were captured using a Zeiss Axiophot microscope fitted with a ProgRes® $C14^{plus}$ / CapturePro 2.8.8 camera.

3.3.6 Fluorescent Intensity Analysis

To determine $\Delta\Psi_M$ and cell injury, the line intensity function of the ImagePro-Plus 7.0 imaging software (Media cybernetics, Silver Spring, MD) was utilized as outline by the manufacturer. Images from selected fields were analyzed without manipulation by randomly drawing non-overlapping lines using the line intensity tool of the ImagePro-Plus software. Each line drawn across the cell measures the intensity (blue, green or red fluorescence) of each pixel along the line. A minimum of 500 pixels were automatically assessed for each cell counted, with each pixel representing 3 possible data points (red, green or blue fluorescent intensity) when three fluorochromes were used (and 2 in the case of dual fluorochrome staining). The data was automatically tabulated by the image analyzer and was easily transferred to an Excel sheet. Before computation in Excel, each individual data was adjusted by subtracting background intensity. To determine the background intensity, a minimum of 500 pixels were analyzed in areas devoid of cells or debris and the pixel intensities recorded. The average background intensity was then

computed in the spread sheet and subtracted from cell intensity values of each pixel analyzed. Subsequently, the desired values corrected for background were subjected to appropriate analysis.

3.4. Statistics

Data from the *in vitro* studies were subjected to parametric statistical analysis using completely randomized statistical one-way analysis of variance (ANOVA) with Levene's test for unequal variances, using Medcalc 12.3.0 software (Mariakerke, Belgium). The students-Newman-Keuls (SNK) test for pair wise comparison (post-hoc analysis) was used to determine the difference between mean values. The probability values (*p*) required to attain statistical significance for all tests, from appropriate negative controls, was set at $P \leq .05$.

3.4 Results

3.4.1 AMB-treated Oligodendrocytes have an increased Caspase-3 Activity

To profile MO3.13 oligodendrocyte apoptotic response to AMB and AME, we employed the NucView ^{TM} 488 caspase-3 substrate nuclear dye in conjunction with MitoView ^{TM} 633 dye for detection of mitochondrial membrane potential ($\Delta\Psi_M$) in

live MO3.13 cells (see Materials and Methods). Figure 3.1a shows AMB treated cells had a significantly higher activity of caspase-3 enzyme at low concentrations of 1 µg/ml when compared to the glucose controls. Only at concentrations of 10 µg/ml did AME-cells show increased caspase-3 activity (data not shown). A similar trend was seen on epifluorescence analysis of MitoView labeled cells (Figure 3.1b). AMB-treated cells had evidence of reduced mitochondrial membrane potential ($\Delta\Psi_M$) when compared to vehicle controls.

3.4.2 Low Concentrations of AMB Activates Caspase-9 but Not Caspase-8 and -10

Having shown that caspase-3 is induced by both drugs, though at different concentrations, we then set out to delineate which apoptotic pathway was likely involved. Using the FLICA (Fluorochrome inhibition of caspases) dyes for caspase-9 (intrinsic) and -10 (extrinsic), we labeled the treated and control MO3.13 oligodendroglia cells as described in the Materials and Methods section. Fluorescent microscopic analysis revealed that AMB (1 µg/ml) induced caspase-9 activation depicted in Figure 3.3 as increased red fluorescence of the FLICA probe. Conversely, similarly treated AME-cells did not appear to activate caspase-9 as depicted by the lack of red fluorescence. Neither AMB nor AME activated caspase-8 (green florescence seen with staurosporine) and caspase-10 (data not shown).

3.4.3 Mitochondrial Cytochrome c Release

To further demonstrate MMP involvement in AMB induced apoptotic injury in MO3.13 oligodendrocytes, we assessed the release of cytochrome *c* after cells were treated. After 10 minutes of incubation with 1 µg/ml of AMB, cells appeared with diffuse fluorescence of cytochrome c similar to the positive controls (Figure 3.3). However, 1 µg/ml AME remained bright and punctuate like the dextrose controls. These results suggest that AMB elicited cytochrome *c* release, consistent with MMP and consequent mitochondrial mediated apoptosis.

3.4.4 Mitochondrial Membrane Potential $\Delta\Psi_M$

Mitochondrial cytochrome *c* release and $\Delta\Psi_M$ loss occur secondary to MMP and serve as predictable markers of irreversible commitment to cell injury and demise by apoptosis (Kroemer et al., 2007). Both events play an integral role in apoptosis. Low concentrations of AMB but not AME appear to perturb mitochondrial membrane permeability as evidenced by cytochrome *c* release.

To confirm the effect of either test compound on the $\Delta\Psi_M$, we employed the mitochondrial membrane potential dependent dual fluorescent JC-1 dye. Healthy dextrose control cells showed predominantly red punctuate fluorescence (Figure 3.4 ABC; Figure 3.5; Figure 3.6).

Figure 3. 1 A Low concentrations of AMB enhances the activity of caspase 3 enzymes

AMB at 1 µg/ml showed a significantly higher activity of caspase 3 enzyme in comparison to 10 µg/ml of AME. Both 1 µg/ml AMB and 10 µg/ml AME showed a significant differential induction of caspase-3 when compared to their respective controls. Each data point represents mean ± SEM of 3 independent experiment (n=3, each the mean of duplicates). *p= < 0.05, ** p <0.001compared to control.

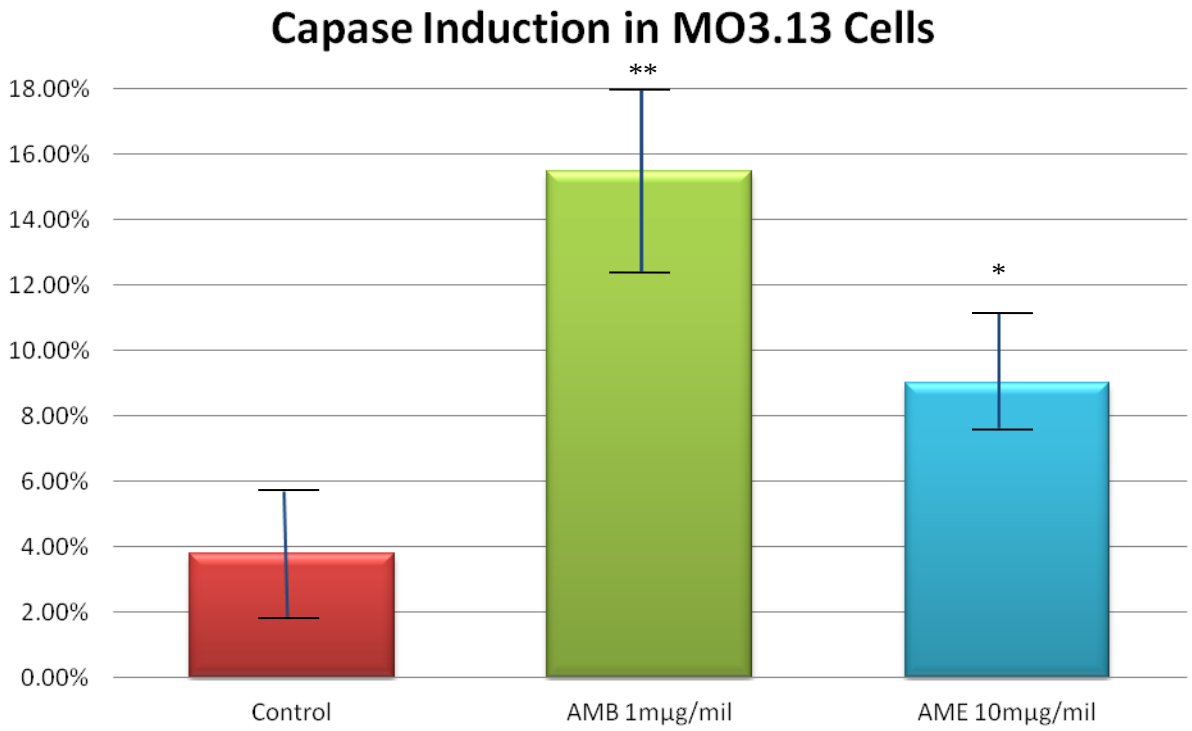


Figure 3.1b Micrographs of MO3.13 glia cells labeled with MitoView label

Cells with diminished fluorescence have low mitochondrial membrane potential ΔΨm. (A) Representative images of controls with bright fluorescence reflective of a healthy mitochondria (B) AME 10 µg/ml with a decreased fluorescence intensity indicating loss of ΔΨm (C) low concentration (1 µg/ml) of AMB with diminished MitoView intensity.

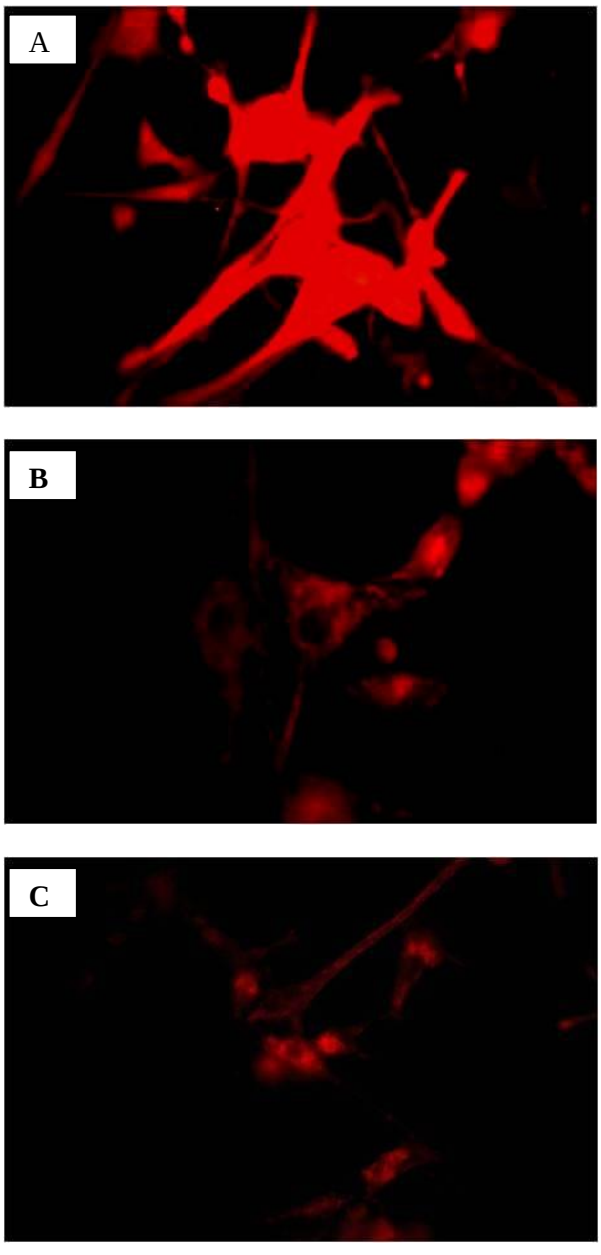


Figure 3. 2 Low concentrations of AMB and not AME induces activity of caspase-9

Caspase-9 activation is reflected by the red labeled fluorochrome inhibition of caspase (FLICA) released in mature human MO3.13 oligodendrocytes (shown above) at 2 hours post treatment. Caspase-8 is demonstrated by green fluorescence is depicted by green block arrows. Magnification is x40

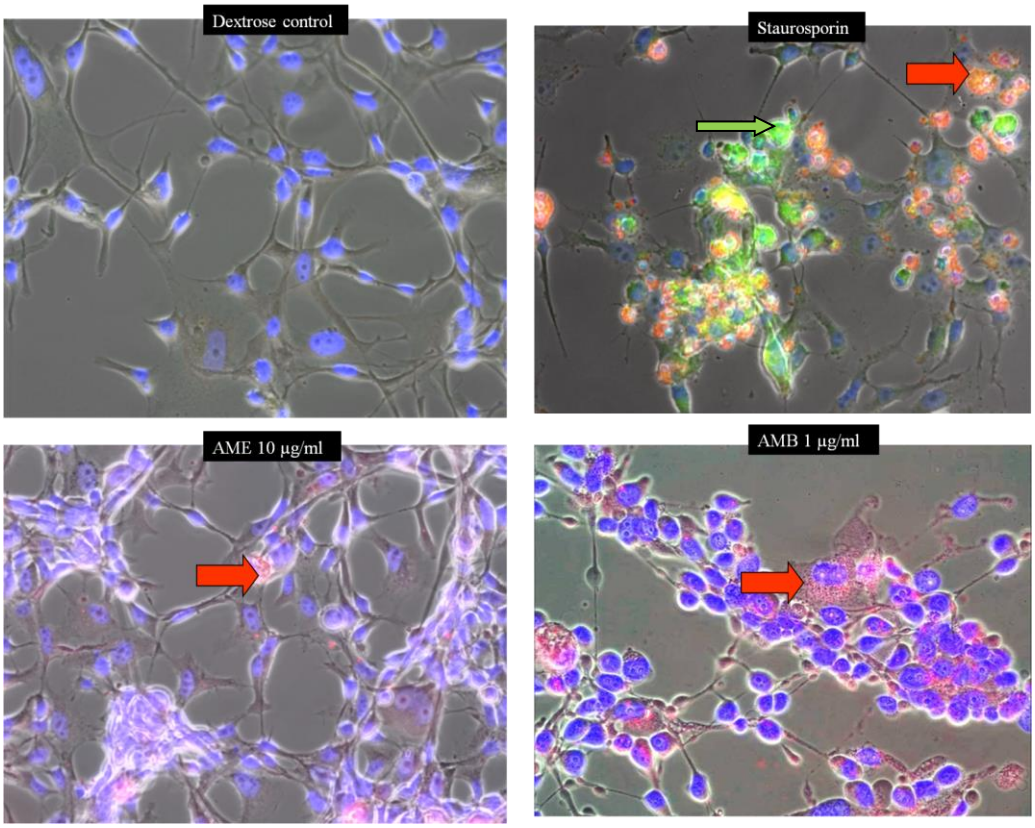
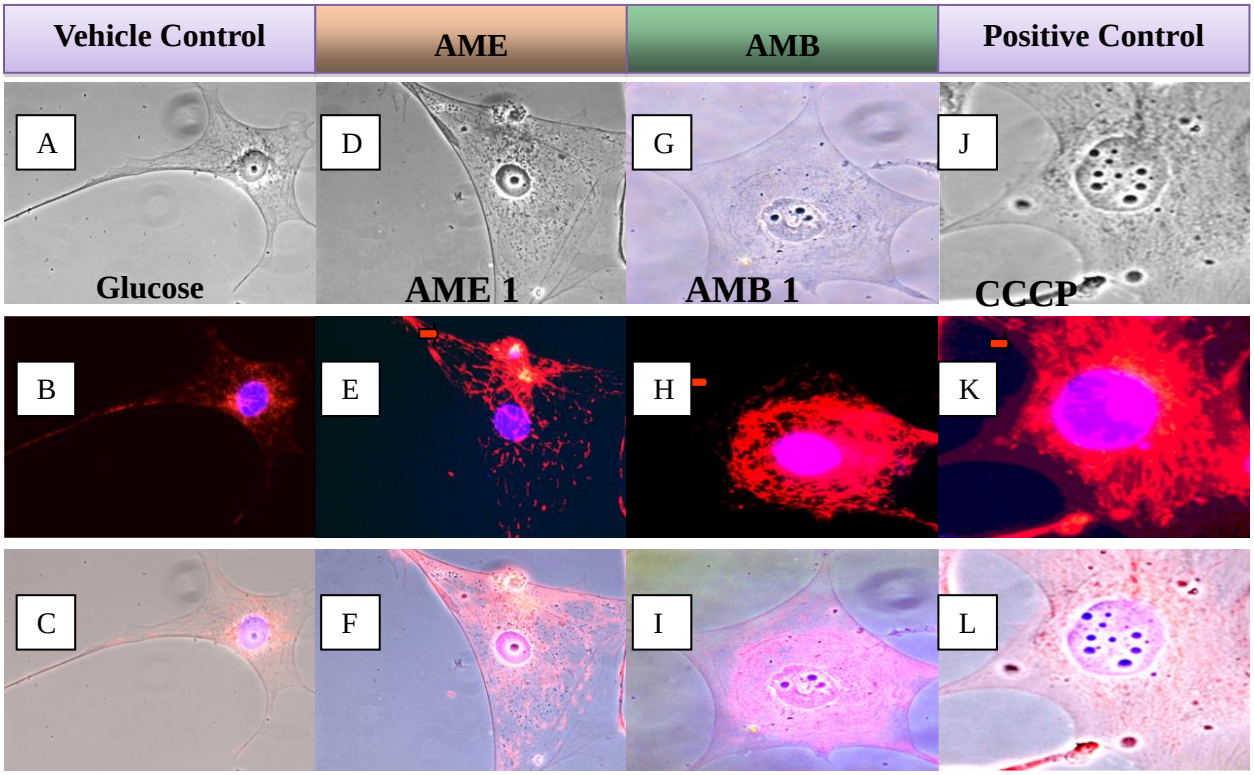


Figure 3. 3 Oligodendrocyte cytochrome c release

(A, B, C) Two hours after exposure, control cells appeared normal with discrete intensely red fluorescent mitochondria indicative of cytochrome c retention within them. (D, E, and F) cells treated with 1 µg/ml AME show similar punctuate cytochrome c fluorescence. However, a diffuse red fluorescence indicating release of cytochrome *c* (G, G, I) is observed in 1 µg/ml AMB treated cells similar to (J, K, L) to the positive control CCCP (carbonylcyanide m- chlorophenyl hydrazone). Panels A-L is representative images depicting the release of cytochrome c from the oligodendrocyte mitochondria. Scale bar is 100µm.



Similar to the controls, 1 µg/ml AME-treated cells had red punctuate fluorescence, indicating a high $\Delta\Psi_M$ and presence of JC-1 aggregates. However, a similar concentration of AMB showed an increased intensity of the diffuse green monomeric JC-1 fluorescence that was consistent with the CCCP positive control. This implied AMB perturbs the mitochondrial membrane as confirmed by a significant decrease in $\Delta\Psi_M$ as depicted in Table 3.1.

3.4.5 Trolox Does Not Reverse AMB Induced $\Delta\Psi_M$ loss

Despite acute Trolox treatment, MO3.13 glia cells incubated with 1 µg/ml AMB did not show significant improvement in $\Delta\Psi_M$ loss (Figure 3.6; 3.7). Trolox, a water soluble vitamin E analog is an antioxidant that prevents loss of $\Delta\Psi_M$ (Figure 3.6 A, B). AMB induced loss of $\Delta\Psi_M$ was unaffected by Trolox. These results suggest that Trolox does not promote rescue of oligodendrocytes from clinically relevant concentrations of AMB-induced $\Delta\Psi_M$ loss in cell culture systems.

Figure 3. 4 Mitochondria Membrane Potential is altered by AMB

Below are representative micrographs depicting alteration in the mitochondrial membrane potential of MO3.13 oligodendrocytes. This was assessed by labeling with the JC-1 (5, 5', 6, 6'-tetra chloro-1, 1', 3, 3'-teraethylbenzamidazolcarbocyanine iodide) dual fluorescent dye. A healthy mitochondrial membrane is shown by the interspersed orange to red fluorescence of J-aggregates found only in the mitochondria with an intact membrane. Conversely, a diminishing red fluorescence with increase in diffuse green fluorescence of the monomeric form of the dye is indicative of dispersal into the cytosol due to a disruption of the mitochondrial membrane (potential). Panels (ADGJ) phase contrast, (BEHK) merged fluorescent images, and (CFIL) merged fluorescent and phase contrast images. (ABC) Red fluorescence of healthy mitochondria in dextrose (vehicle control) incubated cells. (DEF) Depolarization in compromised mitochondria of CCCP (positive control) treated cells. (GHI) incubation with AME at 1µg/ml. (JKL) depolarized mitochondria of the cells treated with 1µg/ml AMB. Cell nuclei were counterstained with blue Hoechst 33342. Scale bar is 100µm.

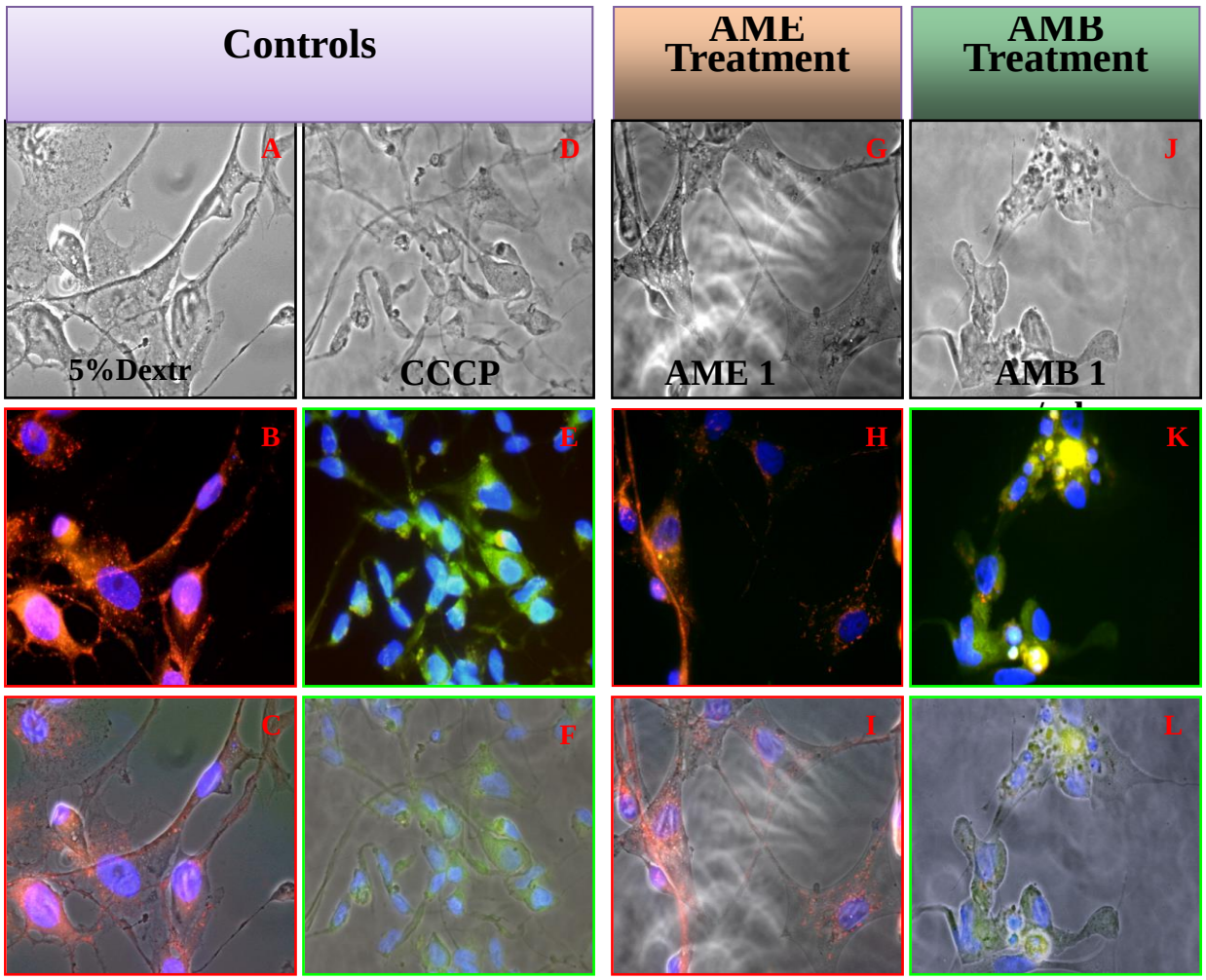

Controls
AME Treatment
AMB Treatment
A
D
G
J
5%Dextr
CCCP
AME 1
AMB 1
B
E
H
K
C
F
I
L

Figure 3. 5 Quantification of mitochondria membrane Potential loss

Representative fluorescent image and software (Image Pro-Plus 7.0) output of line intensity measurement for Jc-1dye.

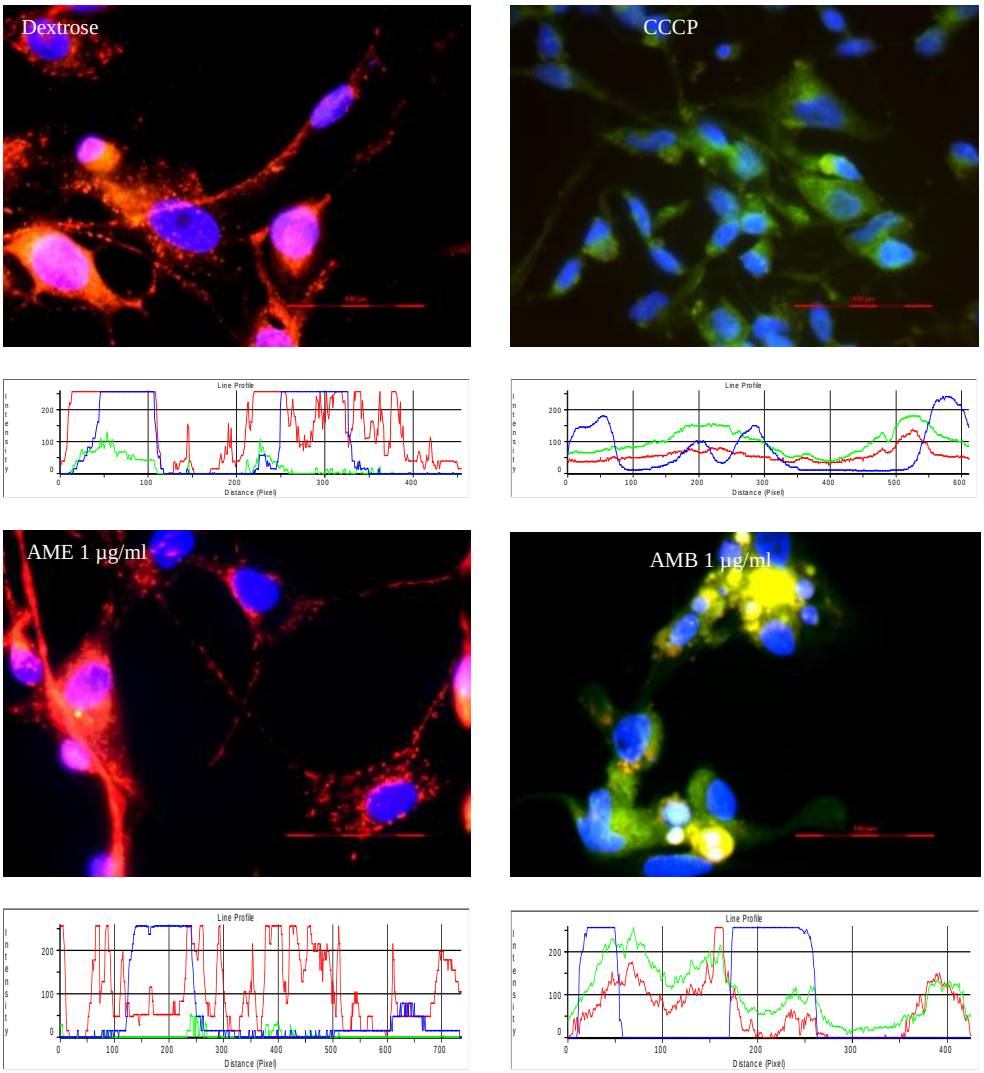


Table 3. 1 Quantification of mitochondria membrane potential loss

The fluorescence intensity values of the JC-1 dye labeled MO3.13 oligodendrocytes were determines using the line intensity tool (Figure 3.5). (F (λ580/F (λ535) is ratio of red fluorescence (High $\Delta\Psi_M$) to green fluorescence (low $\Delta\Psi_M$) after subtraction of background fluorescence. This ratio was only high (high membrane potential) in the vehicle controls and AME 1 mcg/ml treated cells. Each independent experiment (n=3) is the mean of duplicates. *p= < 0.05 compared to vehicle control.

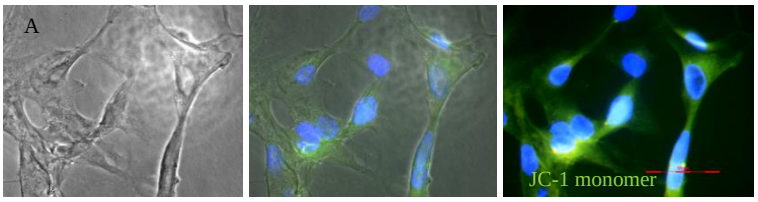
Treatment	**n**	**Mean ratio F (λ580/F (λ535). $\Delta\Psi_M$**	**SEM**
(1) AMB 1 mg/ml	3	0.6188	±0.05620*
(3) AME 1 mg/ml	3	9.4276	±2.3359
(4) CCCP	3	0.8020	±0.2065*
(5) Control	3	10.3820	±1.5341

Figure 3. 6 Trolox does not promote the rescue of cells treated with AMB

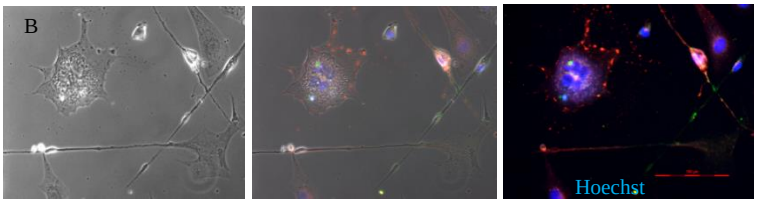
(Panel A) the positive control (CCCP) treated cells demonstrate loss of membrane potential (green), this loss of potential ($\Delta\Psi_M$) is markedly reduced (panel B) in cells pretreated with Trolox. (Panel C and D) In contrast, dextrose controls retain punctuate red/orange fluorescence of viable mitochondria that promotes JC-1aggregate formation. (Panel E and D). Despite pretreatment with Trolox, mitochondria of AMB treated cells were not significantly protected from loss of $\Delta\Psi_M$ (green), contrasting (Panels F and G) cells exposed to high concentration of AME which demonstrated significant reversal of loss of potential. Scale bar is100 µm.

Phase contrast (PC) Merge Fl+PC Merge (Fl)

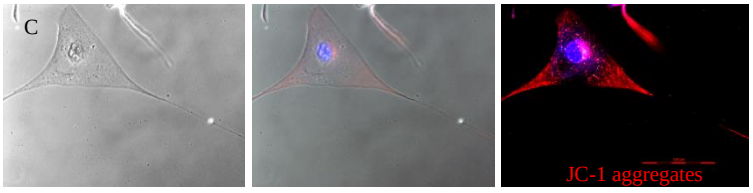
MPTP-JC-1 dye + Trolox Rx



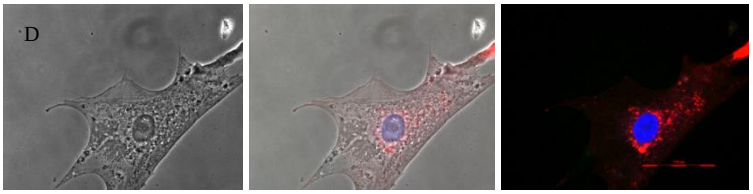
CCCP



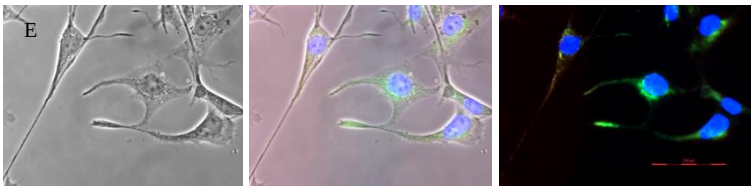
CCCP + Trolox



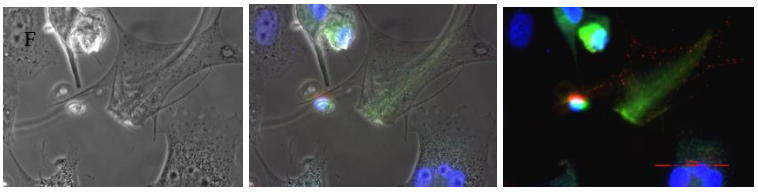
Dextrose control



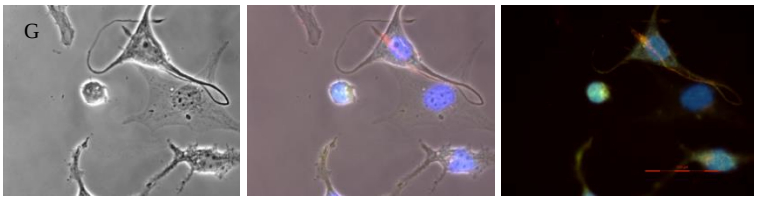
Dextrose +Trolox



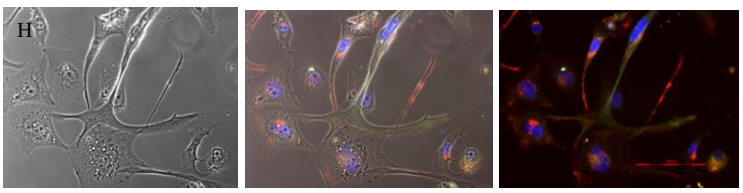
AMB 1 µg/ml



AMB 1µg/ml+Trolox



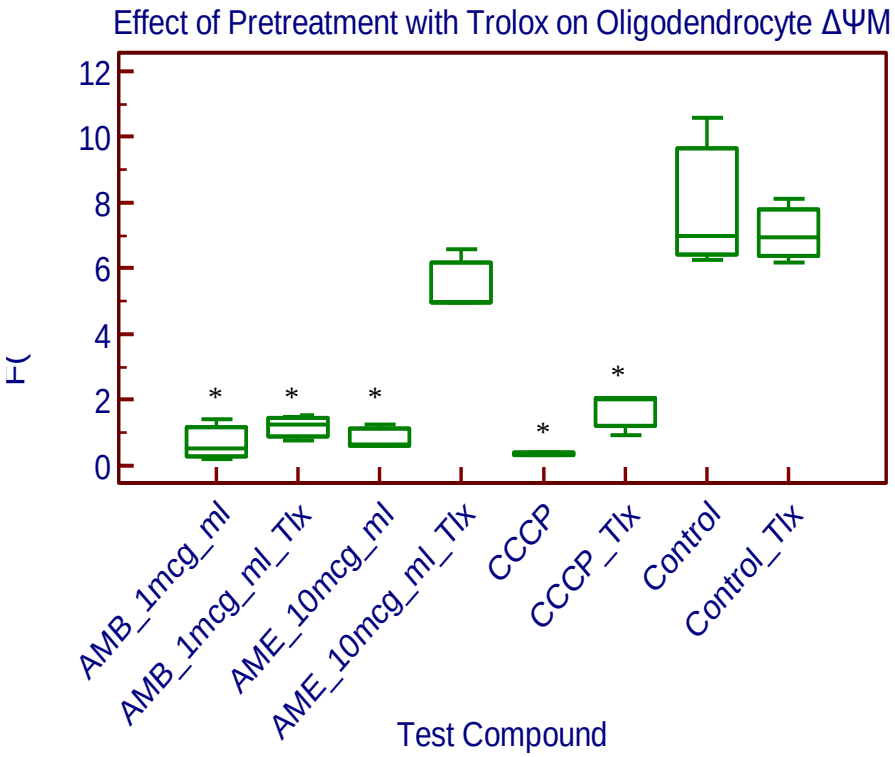
AME 10 µg/ml



AME 10 µg/ml+ Trolox

Figure 3. 7 Trolox does not Reverse Loss of $\Delta\Psi_M$ due to AMB

Images of live cells labeled with JC-1 dye in Figure 3.6 were analyzed using Image Pro-Plus 7.0 analysis software. Six random fields per slide were chosen and the intensity of the desired dye was determined. The ratio of red fluorescence (F(λ580), high $\Delta\Psi_M$) for each treatment group to that of green fluorescence (F(λ535**),** low $\Delta\Psi_M$) was expressed as the average +/- SEM of 3 independent experiment (n=3, each the mean of duplicates). **p*= < 0.05 compared to respective vehicle control. Scale bar = 100 µ



3.5 Discussion

This present study was performed to determine if AME and AMB induced mitochondrial membrane permeability (MMP). Our data suggests the mitochondria participate significantly in the injury of oligodendrocytes in a manner consistent with activation of the intrinsic but not extrinsic apoptotic pathway. To our knowledge, this is the first report to provide evidence that AMB induces loss of mitochondrial potential in oligodendrocytes.

3.5.1 AMB induces Caspase-3 activation

Our current study showed that oligodendrocytes undergo apoptotic phenotypic changes when treated with AMB. The NucView^{TM} 488 caspase probes we used allowed us to monitor the fluorescent signals in whole-living cells with active caspase enzymes. On epifluorescence microscopy, we observed an elevated caspase-3 enzyme activity in the cells treated with 1 µg/ml AMB. This was depicted by increased fluorescence of NucView^{TM} 488 label. Notably, we demonstrate that AME-cells at similar concentrations do not bear activated caspase enzymes. Several reports have shown that initiator caspases exist and can be activated by various stimuli, ultimately, leading to the activation of the effector caspases caspase-3. Activation of effectors caspase-3 can occur after depolarization of the mitochondria (Inai et al., 1997) leading to the characteristic changes

associated with apoptosis, like cell shrinkage, DNA fragmentation and membrane phosphatidylserine externalization (Li et al., 1997). It can also occur after release of inducer caspases-9 (intrinsic) -8 and -10 (extrinsic).These data lead us to infer that the nuclear morphological changes indicates apoptosis in the preceding *in vitro* studies resulted from AMB.

Chemical rescue of cells undergoing apoptosis is possible. However, one draw-back with the current experiment was with the use of caspase-3 inhibitor II. Pretreatment of cells with the inhibitor led to significant detachment of cells treated with AMB and AMB. Although some cell detachment was apparent with the controls, it was less than that observed with AMB and AME. Further investigation without antibiotics (streptomycin) resolved the problem, implying that streptomycin which also has a macrolide ring like the polyene test antibiotics may be confounding the cell culture experiments. We elected to carry out all cell culture experiments and repeat experiments devoid of antibiotics. Nevertheless, the current experiments could not be completed because of insufficient material. Logistic challenges from growing the cells without antibiotic cover made it labor and capital intensive.

3.5.2 Amphotericin B Activates the Intrinsic Apoptotic Pathway in Oligodendrocytes

The current results support earlier reports that AMB toxicity can be mediated by apoptosis. Varlam et al (2010) demonstrated AMB causes apoptosis in kidney cells in

culture as well as in mice. We have demonstrated that AMB induces activation of caspase-9 enzyme. Although not always, activation of caspase-9 is dependent on the mitochondrial release of cytochrome c with consequent cleaves and activates caspase-3, the downstream effector of apoptosis (Thornberry and Lazebnik, 1998). Caspase-8 and -10 were refractory to AMB's effect. AME had no effect at low concentrations on the three enzymes. The fact that AMB activated caspase-9 but not -8 and -10 suggests that it may act via the mitochondrial intrinsic pathway to execute its apparent apoptotic effects. It is unclear why the caspases are insensitive to AME which is closely related to AMB, but is likely that intrinsic physiochemical differences may play a role in the disparate sensitivity.

3.5.3 AMB Treatment Causes Cytochrome *c* Release in MO3.13 Cells

Cytochrome c is an electron carrier in the mitochondrial electron transport chain. Electrostatic forces bind it to the exterior of the inner mitochondrial membrane (Poppe et al., 2001; Haq et al., 2004), and early experiments showed that cyt *c* was involved as an early step with the activation of caspases accompanied by mitochondrial membrane potential depletion (Von Ahsen et al., 2000). Although the mechanism of how cyt c is released and its importance in neurotoxicity are largely unclear, accumulating evidence shows that secondary to diverse insults, various mechanisms may lead to the mitochondrial escape of cyt c (Lieven et al., 2003).

We demonstrate that cytochrome *c* can be released by low concentrations of AMB. With AME, we observed the immunofluorogenic appearance of cytochrome *c* as bright punctuate fluorescence in the cell, indicating its mitochondrial localization implying healthy oligodendrocyte cells. However, it appears as a diffuse fluorescence when released to the cytosol, such as seen with similar concentration of AMB. This supports our conclusion that AMB may act via a central mitochondrial pathway to mediate injury to oligodendrocytes.

3.5.4 Diminished $\Delta\Psi_M$ is Seen with AMB Exposure

One of the earliest biochemical events of cell death, whether by apoptosis or necrosis, is dissipation of the potential across the inner mitochondrial membrane and formation of nonspecific pore by the adenine nucleotide translocase (ANT) (Halestrap et al., 2002) or mitochondrial permeability transition pore (MPT). The mitochondrial permeability transition event (MPT; $\Delta\Psi m$) (Hunter and Haworth, 1979) involves the opening of channels in the inner mitochondria membrane, allowing free distribution of solutes between matrix and cytosol (Bernardi, 1999).

Based on our current results from MitoView fluorescent labeling(Figure 3.13), AMB may perturb $\Delta\Psi_M$. To confirm, we employed a more sensitive method, the JC-1 dye (see Materials and Methods) to assess to the mitochondrial injury with the possibility of quantifying $\Delta\Psi_M$. We demonstrate a significant reduction of $\Delta\Psi_M$ that was more

severe for AMB (1 µg/ml) than AME (10 µg/ml). AME at 1 µg/ml was clearly not toxic to the mitochondrial membranes.

The work of Douglas and colleagues (2010) is in accord with our data on AMB. The mitochondrion is known to have a lipid-rich outer membrane. Although it is presently unclear if AMB or AME induces pore formation in membrane-bound organelles such as mitochondria, the claim of several workers that AMB is internalized into mammalian cells because of it high affinity for lipids (Volmer and Carreira, 2010) lends support to this hypothesis. If this is so, it may in part explain the differential potencies of AMB and AME as their ability to bind directly to the mitochondria and create ion channels differs.

3.5.5 Trolox Does Not Prevent $\Delta\Psi_M$ Loss in Cells Treated with AMB

The MPT pore is a complex that consists of several proteins of the inner and outer membranes such as ANT (Halestrap et al., 2002). These proteins are potential targets for chemical modulation. For example, the MPT channel blocker cyclosporine A (CsA) is proposed to sit in a hydrophobic pocket of CyP D (Basso et al., 2005) and inhibits $\Delta\Psi_m$. While bongkrekic acid inhibits ANT keeping the pore closed (Henderson et al., 1970). The water-soluble anti-oxidant Vitamin-E analog Trolox (6-hydroxy -2, 5, 7, 8 – Tetramethylchromane 2-carboxylic acid is efficient at preventing proton leaks in brain mitochondria (Brookes et al., 1998).

Our results provide evidence that acute Trolox pretreatment did not prevent $\Delta\Psi_M$ loss in oligodendrocytes due to AMB (1 µg/ml). Acute Trolox treatment did not modulate AME-cells nor did it change $\Delta\Psi_M$ in normal cells (glucose controls). Our data was not robust enough to draw strong inference on the role of Trolox rescuing loss of $\Delta\Psi_m$ AMB-cells. A time-course or rigorous recovery study that will incorporate a chronic Trolox rescue regimen will improve the experiment.

Although transient opening of the pore, leads to the release of cytochrome c and other pro-apoptotic proteins (Debatin et al., 2002), it is the downstream events which ultimately lead to cell injury and death (Susin et al., 1997). The downstream effects following loss of $\Delta\Psi_m$ such as caspase enzymes activation with consequent nuclear changes consistent with apoptosis following low concentrations of AMB suggests mitochondria involvement in cytotoxicity. Mitochondria are rich in lipids and this may account for its vulnerability to AMB attack.

To maintain the activity of ATP synthase, the mitochondrial membrane is specialized to be impermeable to protons, leading to the creation of a trans-membrane potential (Dilda and Hogg, 2005). Loss of $\Delta\Psi_m$ as seen with AMB has dire consequences for cellular process due to loss of ATP. Mechanistically, if AME does not modulate $\Delta\Psi_m$ then it presents a potential therapeutic adjunct to AMB in the fight against systemic fungal infections.

Chapter 4

4 General Conclusions and Future Directions

4.1 Introduction

The rise of fungal infections as an important cause of blood stream infections makes it imperative to develop new agents or discover new ways to use existing agents to combat the complex disease. In addition, it is equally important to understand their modes of action to ensure their safe use. Various mechanisms have been suggested to explain how AMB and its many congeners such as AME act; however, there is consensus on one: that they form a barrel like trans-membrane channel with consequent loss of vital cell content cell lysis. Nevertheless, this mechanism does not completely account for AMB's heightened neurotoxicity, nor does it fully account for the absence of neurotoxicity with AME. Moreover, it has been suggested that this channel formation, particularly with AMB may mask other mechanisms that coexist (Volmer and Carreira, 2010).

The current study explored the neurotoxicity of AMB, the commonly used antibiotic for treating severe deep-seated fungal infections, and compared it to its purportedly effective analog, AME. The current study has identified relative roles of both drugs in neurotoxicity. Furthermore, the study sought to understand the molecular mechanism(s) of how AME, relative to AMB, affected the identified targets, and

explored if chemical protection can lead to reduction in potentially observed toxicity. Literature on the safety of AME relative to AMB, particularly regarding AME's chronic intravenous administration and reputed neurotoxicity in its early human trial is sparse, and leaves many important questions unanswered. The current study was designed in an effort to answer some of these lingering questions:

a) Are oligodendrocytes differentially sensitive to AME and AMB *in vitro*?

To answer this research question, the key experimental findings from each Chapter (*1, 2, 3)* will be highlighted.

4.2 Is AMB more toxic to differentiated MO3.13 human oligodendrocytes cell-line than highly purified AME *in vitro*?

The main findings that address the research question were specific to two experimental chapters, 1) *In vitro* toxicity of amphotericin B and amphotericin B methyl ester to mature oligodendrocytes, and 2) *In vitro* mitochondrial membrane effects of AME and AMB.

Our finding in the current study is consistent with sterol hypothesis that polyenes alter cell membrane permeability consequently leading to cell death (Bolard, 1986; Hammond et al., 1974; Hammond, 1977). AMB caused increased cell membrane permeability in oligodendrocyte cells in culture, consistently causing membrane blebbing and vacuolations in unstained cells that were not seen with AME. Our study also provides *in vitro* evidence that AME concentrations that were otherwise non-toxic may become toxic when small amounts of AMB are added. This is consistent with the data from Reuhl

et al (1993). However, the question arises as to why AMB is not universally neurotoxic in humans if small amounts can evoke injury to oligodendrocytes

These preceding observations added to the data from literature on the mechanism of AMB and AME prompted a dye exclusion test that was initially performed using trypan blue. The results suggested a dose-dependent cytotoxicity with both drugs. However, the lowest dose of AMB to cause toxicity was 1µg/ml at 2 hours compared to 10 µg/ml for AME. This suggested that low concentrations of AMB can lead to necrotic death while the oligodendrocytes appeared to be resistant to low concentrations of AME (Racis et al., 1990). However, the fact that most of the cells treated with low concentrations of AMB that stained with trypan blue were still attached to the slides (coverslip) suggested necrosis may not completely account for their cell death.

To confirm this thinking, fluorescent microscopic examination of propidium iodide and fluorescein diacetate (PI/FDA) stained mature oligodendrocytes confirmed that they were indeed sensitive to AMB cytotoxicity at low doses as shown by the significant background PI staining of the nucleus. However, significant numbers of cells in the low concentration group of AMB were FDA^{+} with high background of PI, implying the cells were still alive even though their membranes were compromised. One possibility could have been that the dying cells were in the early phase of necrotic death. However, the observation of a background morphological change reminiscent of apoptosis suggests otherwise. The apoptotic nuclei were distinctly stained with PI. This observation was in support of our in vivo results that AMB but not AME can induce apoptotic change in CNS cells such as oligodendrocytes.

From a mechanistic point of view, our study could not discern which came first: the membrane permeability or the apoptosis. As such it was unable to answer the question if apoptosis was secondary to the membrane pore formation. A rigorous time lapse fluorescent microscopic assessment that incorporates a patch clamp techniques may resolve this question. What the current study was able to do was confirm the apoptotic change using the nuclear area factor (NAF). The NAF is a reliable, reproducible and sensitive method for assessing very early changes in apoptosis (DeCoster, 2007; Afifi et al., 2013).

The activation of the group of cysteine protease enzymes (caspases) that recognize specific aspartate residues in target proteins is a distinctive biochemical feature of apoptosis (Nicholson and Thornberry, 1997). AMB-induced apoptosis in oligodendrocytes as demonstrated in this study also was seen in several *in vitro* systems (Varlam et al., 2001; Mousavi and Robson, 2004), supporting our original hypothesis that AMB, but not AME may induce cytoxicity in oligodendrocytes.

We observed in our current *in vitro* studies that AMB elicits changes consistent with apoptosis in oligodendrocytes, but similar lesions were not observed with AME. This is important not only because it reaffirms the good safety profile of AME, but also because it indicates that the difference in apoptosis by AMB relative to AME in brains of mice is not merely due to pharmacokinetic and homeostatic considerations, since the cell culture experiments circumvents all distribution effects.

As observed in this study mitochondria may be a possible target of AMB. In fact, 10 min after exposure to AMB, oligodendrocytes were noted to release cytochrome *c* into

the cytoplasm. Cytochrome *c* has be shown to be apoptosis-specific and its release from the inner mitochondrial membrane into the cytosol signals an early event in the mitochondrial mediated pathway of apoptosis involving procaspase-9 (Slee et al., 2001). This downstream effect of mitochondrial membrane perturbation is consistent with our results using AMB but contrasts with AME which had no detectable effect on cytochrome *c* release.

The activation of caspase -9, but not -8 and -10, lends support to the suggestion that mitochondria initiator caspases-9 activation reflects mitochondrial intrinsic pathway activation (Thornberry and Lazebnik, 1998). Accordingly, AMB, but not AME, revealed a mitochondrial mediated apoptosis with $\Delta\Psi_M$ loss. We demonstrate that $\Delta\Psi_M$ in oligodendrocytes was significantly reduced by AMB treatment but not by AME, implicating mitochondrial membrane permeability (MMP) and suggesting MMP and consequent downstream effects.

The biochemical cascade of apoptosis is amenable to regulation (Craighead et al., 1999). Bearing this in mind, we observed that oligodendrocytes were partially protected from the loss of $\Delta\Psi_M$ by AMB with Trolox treatment, suggesting that AMB-induced loss of $\Delta\Psi_M$ might be redox-sensitive and is mediated at the mitochondrial membrane level. It is unclear why AME, which works through a similar formation of pores, is not myelinotoxic. The answer may lie in critical variables discussed in preceding sections.

The sterol pore model of polyenes action proposes cell membrane ion channel formation as the mechanism of AMB (and AME) biological activity (Hartsel et al., 1992). This is consistent with the observations from this study with regard to both AME and

AMB. Nevertheless, what was observed in this study was that an alternate pathway with the mitochondrial membrane playing a role may exist for AMB with consequent apoptosis. This mechanism of AMB toxicity *in vitro* could account for the apoptotic change seen in the white matter of mice.

4.3 Is AMB more toxic to myelin than highly purified AME *in vivo*?

The literature revealed that nearly all studies using less than 1.3% AMB content in their AME preparations reported no neurotoxicity with AME use. This is important because in the Hoeprich human trials with AME, all the patients who received AME had been recruited based on the criteria that their prolonged AMB treatments had failed, or that they were suffering from AMB-induced nephrotoxicity (Hoeprich et al., 1988). Thus, apart from AMB contamination in the low purity samples used in the trials, there is a very real possibility that the neurotoxicity observed in the AME trials may have been due, in part, from prior AMB overdose and solely (or even significantly) from the AMB content.

Evidence supporting the premise that AMB is neurotoxic has been provided by investigational neurotoxicity studies, just as human clinical data has long implicated it as a cause of neurotoxic side effects. Conversely, accruing data show the adult brain is less vulnerable to highly purified AME. Put together, this led us to hypothesize that myelin membrane (and oligodendrocytes), a major target of tainted AME and AMB injury in the

adult CNS, would be more prone to AMB than pure AME. Since the potential neurotoxicity of AME has not been compared to AMB in a murine model, we initially sought to evaluate their general toxicity since the literature suggests AMB's general toxicity is significantly higher than AME's across most species of experimental animals.

Our clinical findings support the work of several investigators indicating that AMB causes significant systemic and organ toxicity, contrasting AMEs lower toxicity (Table 2.1). Adult mice doses i.v with 5 mg/kg b.w of AMB or AME for one month led to a weight gain in AME animals, with no deaths or CNS effects recorded. The notable findings were the AMB-induced weight loss after a month that was associated with death and notable lethargy and irritability in some of the animals. This trend of systemic toxicity was observed long after cessation of treatment and only in the AMB group. The reason for the presence of systemic toxicity in only the AMB groups is not clear.

Quantitative structure activity relationship (QSAR) predictions offer an explanation, suggesting that the lack of a free carboxylic acid (COO^-) in the AME molecule conferred on it a high fidelity to discriminate between mammalian cholesterol and fungal ergosterol membranes (Chéron et al., 1988). If this postulate is true, then AMB with a free carboxylic acid group will have a relatively poorer ability to differentiate between the two membrane types than AME. Alternately, it is postulated that AMB's self-association into aggregates is essential to its mechanism of action and thus toxicity. Some investigators have reported that water soluble AMB-aggregates may be solely responsible for AMBs action on cholesterol membranes (Volmer and Carreira, 2010). They further suggest that both the monomeric and water-soluble aggregates are responsible for its action on ergosterol, giving additional clues as to why AMB impacts

the fungal pathogen more than it does its human host. If indeed the preceding views are valid, then it means that AME, which is not reported to self-associate, is less likely to affect cholesterol membranes relative to AMB.

The perception in medical literature that AMB is less neurotoxic than AME needs to be re-examined, particularly as highly purified formulations of AME are now currently available. Our current study contradicts this perception, and supports the premise that AMB content of the AME preparations used in its flawed trials and early *in vivo* neurotoxicity studies investigations led to the purported white mater injury. Indeed, our histological and fluorescent microscopic findings after a month of treatment suggested that the cerebral white matter may not be vulnerable to AME toxicity. The fact that AMB- cerebral white matter showed signs of myelin damage reminiscent of those observed with tainted-AME supports the claim that AMB as a contaminant of the AME lots used by the investigators was responsible for the alleged neurotoxicity associated with AME use. We did not observe a marked astrogliosis as reported by the various investigators that employed tainted AME or AMB, perhaps implying a reduced inflammatory response. Alternately, this could mean lower doses of AMB reached the brain parenchyma; both drugs are reported to penetrate the brain poorly.

However, AMB apparently induced nuclear apoptotic changes in the areas we detected demyelination. This could account for why we did not detect remarkable astrocytic and microglial reaction with the AMB-brains. The morphological changes in the nucleus appear to be limited to non-neuronal cells, implying that glial cells (oligodendrocytes, microglia or astrocytes) are the target of this apoptotic nuclear change. While there is a paucity of reports suggesting AMB or AME causes apoptosis in the

brain, AMB can cause apoptosis in mammalian kidneys (Varlam et al., 2010). It is unclear if the apoptotic change is a secondary event to the membrane permeabilzation mechanism of AMB. However, if this was the case, AME sharing the same mechanism should have elicited a similar morphological change.

The experiment assessing for myelin damage after 2 month-alternate day injections of 5 mg/kg AME and AMB, followed by a 5-month cessation produced notable changes in adult AMB-mice but not in AME-mice. The myelin loss as indexed by diminished fluorescence intensity of fluoromyelin was observed in the sub-cortical white matter of the cerebrum. We observed that many cells that appear to engulf myelin debris were present, suggesting gliosis and consequent inflammatory reaction. This was associated with a reduced nuclear morphological change that was seen previously in the 1-month study.

The literature provides no experimental precedence to compare neuropathological findings after sub-chronic administration followed by chronic cessation of either drug. However, some investigators like Gold, Ryzlak and Schaffner (1990) provided clues by demonstrating myelin and axonal injury in sciatic nerves of rats that was more severe with AMB than with AME and persisted after cessation of treatment. Indeed, consistent with our findings in the brains of mice, they described a poor remyelination process and stunted axonal regeneration with the AMB-rat sciatic nerves of rats. Similarly, their evidence supports our speculation previously that repeat dosing of AMB initiates a process of white matter injury, which gains momentum, propagating a progressive injury to myelin that is slow to reverse despite chronic cessation of therapy

The literature suggests that differential neurotoxicity effect observed *in vivo* between AME and AMB imply that several important variables may exist that modulate the host response to both drugs. The intrinsic physiochemical properties, route of administration and pharmacokinetics of both drugs are important modulators of their neurotoxicity just as (Racis et al., 1990; Reuhl et al., 1993), others key considerations are route of administration, individual animal variability and host neural response to injury.

Although AME and AMB have a close structural similarity (Schaffner and Mechlinski, 1972; Mechlinski and Schaffner, 1972), evidence from chemical studies signify that the various physiochemical differences between the two drugs for example, a low aqueous solubility confers on AMB (0.0017 mg/ml) a greater toxicity relative to its more soluble analog AME (75 mg/ml). Other physiochemical considerations such as molecular weight, lipophilicity, cerebral blood flow and plasma protein binding need to be taken into account. The route of administration may play a key role on how the drug is distributed and how the host handles the drug.

We administered both drugs via the intraperitoneal route, thus the drugs distribute to the tissue compartment first, which may result in a delay to reach steady -in the plasma since both drugs when distributed to tissue compartment have a slow return phase to the plasma compartment. In addition AMB binds avidly to plasma lipoproteins in sharp contrast to AME, entertaining the possibility that it may also bind to peritoneal fat which may in turn act as storage. If this view is correct, this may present a risk if the animal progressively loses weight as is often seen with AMB therapy, with consequent release of AMB from the lipid depot.

Several studies have demonstrated that the distribution of AMB or AMB to the CSF is poor after systemic administration. However, assay of brain parenchymal levels in non-infected rabbits shows that AMB can get into the CNS (2-27% of plasma levels) and infections can increase this two to four-folds (Kethireddy and Andes, 2007). This indicates that significant amounts of AMB may get into the brain of healthy animals. The fact that Reuhl and coworkers (1993) were able to provide evidence of AMB cytoxicity towards perivascular pericytes lends support to the premise that cumulative doses of AMB can compromise the BBB in health animals. If this is the case, it may account for the difference we observed in toxicity of both drugs. How AMB gets to the brain is unclear. Since both drugs have large molecular weight, to get into the brain they have to traverse the very tight junctions linking the epithelium of the cerebral endothelium as well as that of the choroid plexus, both of which make up the blood brain barrier (BBB). In addition, the endothelium has no aqueous pores, such that both drugs have to be lipid soluble to traverse the membranes (Kethireddy and Andes, 2007). It is difficult to account for how such large molecules get into the brain, unless there is an influx carrier. Nonetheless, individual biological makeup and variation may account for the disparity in tissue distribution between the two drugs.

One consistent histological feature of AMB and AME contaminated with AMB is astrogliosis with activation of macrophages (Hoeprich et al., 1985; 1988; Ellis et al., 1982; 1988; Reuhl et al., 1993). We observed in our 2-month study with cessation of therapy, cells that appear to engulf myelin debris, reminiscent of microglia invasion. It is unknown if AMB or even AME stimulates mononuclear phagocytes (macrophages) in peripheral blood or and microglia in the CNS to produce soluble factors that are toxic to

myelin. Microglia responds to noxious stimuli by AMB by producing pro-inflammatory cytokines and nitric oxide that are toxic. AMB induces the microglia to down-regulate the protective cilliary neurotrophic factor (CTNF) and neurotrophin-3 mRNA expression (Motoyoshi et al., 2008). The microglia enhances cytokine production that is suggested to stimulate astrocytes to produce neurotoxic factors (Trajovic et al., 2001). However, the microglia in parallel responds by increasing production of the neuroprotective interleukin-1 beta (IL-1-β), interleukin-6 (IL-1-β) and brain derived neurotrophic factor (BDNF).

Preliminary *in vitro* work in our lab suggested microglia produced cytotoxic factors (nitric oxide) after AMB exposure. Further preliminary *in vitro* work in our lab suggested non- myelinated neurons were insensitive to both drugs. Although the preceding variables may account for the differential toxicity of both drugs observed, the current study was unable to draw strong inferences on the relative myelin toxicity of AMB and AME due to insufficient number of animals. In addition, due to species differences and the variability introduced by the route of administration that was different from that used in humans, caution should be exercised when interpreting these results.

At this point we elected to examine the direct effect of both drugs on an appropriate cell culture model the human MO3.13 oligodendroglia cell line to address some of the remaining question, in an effort to demonstrate with certainty the role of each drug in causing neurotoxicity.

4.4 Implications for the Reintroduction of AME

The clinical trial of AME was heralded as a breakthrough in the fight against severe systemic fungal infections. Unfortunately, a single pathology report from a confounded clinical trial (Ellis et al., 1982) became the basis of its embargo from human use by the FDA (Hoeprich et al., 1982; 1988). Nevertheless, accumulating evidence from experimental neurotoxicity studies (Niehart et al., 1989; Racis et al., 1990; Reuhl et al., 1993), including this thesis, posit that the AME contamination by AMB impurities found in all the lots used for the trials contributed to the adverse clinical outcomes.

The experimental justification of AME's human safety advocates for the review of the FDA's stand on AME, which will enable the use of similar high-grade formulations that are currently available due to technological advancements to rigorously reassess AME safety profile, with particular emphasis on its neurotoxic potential in humans.

Furthermore, our empirical results, along with emerging literature, suggest a potential alternative or adjunct antifungal polyene antibiotic to the very toxic AMB that can be used on outpatient-basis devoid of the strict hospital-based monitoring required for AMB. AMB overdose is difficult to manage because of its high tissue and lipoprotein binding, coupled with its inability to traverse dialyzing membranes. The fact that AME is water soluble with insignificant protein and tissue binding makes potential overdose less likely and management by dialysis easy. Indeed, with this favorable safety profile, AME may yet find utility in chemotherapy of diverse disease states.

4.4.1 Fungal infections

Disseminated fungal infections have become a major cause of mortality worldwide owing to the global HIV pandemic (Armstrong-James et al., 2014). My personal experiences with AMB therapeutics as a physician working in the developing world where poverty and disease complicates access to effective and safe medication, has given me firsthand experience of the difficulties faced with combating disseminated fungal infections. Infections presenting as fungal meningitis are almost universally fatal in poor countries. AMB in the main treatment for the meningeal fungal disease and, even when therapy is successful, it may be debilitating. Moreover, safer lipid formulations and the new classes of antifungals are very costly in developing countries. One attractive option is improve availability of safer and cheaper formulations of AMB (Armstrong-James et al., 2014), such as AME (Hartsel and Bolard, 1996).

Stevens et al (2007) suggested that the newer class oral triazole agents with an extended-spectrum, such as posoconazole, be considered as key treatment for coccidioidomycosis (Valley fever), a conclusion not universally shared. Apart from the side effects of current therapies for Valley fever, they are associated with very high relapse rates, especially with the highly lethal CNS disease (Oldfield et al., 1997; Crum et al., 2004). Moreover, the cost of therapy with these current antifungals drugs is prohibitive (Armstrong-James et al., 2014), as exemplified by the $19,000 PA estimated for life treatment of a gorilla with posoconazole at the Los Angeles zoo (Associated

Press, 2014). These observations support the need for an increased effort to discover safer, cheaper effective alternatives.

This study used its findings to show that AME is a safe drug and the reputed neurotoxicity of impure AME was likely due, at least in part, to its parent drug AMB as a contaminant in the earlier preparations used. This indeed, warrants a policy review by the FDA to enable a structured rehabilitation of AME, targeting a rigorous safety evaluation that includes non-human primate models with subsequent well designed randomized controlled clinical trials to uphold its reintroduction.

4.5 Limitations and Recommendations for Future Research

The debate on the relative neurotoxicities of AME and AMB points to the consequences of hastily performed and poorly monitored clinical trials, regardless of their humanitarian intent. To develop strategies and targets that will enable the reintroduction of AME into clinical use and safer ways of using AMB (and it newer lipid-formulations), it is imperative that more experimental studies are initiated to systematically examine AME's safety at the molecular, tissue, organ and systematic levels. Rigorous preclinical safety evaluation will further knowledge on how pure AME acts on normal CNS tissue, in turn creating a deeper understanding of how to use it safely in clinical practice.

This dissertation has explored the neurotoxic safety of AME, a potentially important antifungal agent, then compared it to the commonly used antifungal agent

AMB *in vitro* and *in vivo*. Caution should be exercised when extrapolating these results due to unavoidable challenges resulting from the study design.

First, are the complications inherent in the use of cell culture systems. Direct administration of test agents to purified cell populations necessarily circumvents the possible interactions of multiple cell types, such as neurons and microglia, which are known to respond via cell-cell cross-talk.

Second, the use of two types of antibiotic preparations in some of the cell culture experiments may have led to overestimation of some results. This may account for some of the variability seen in the study with caspase enzymes. One antibiotic preparation contained streptomycin and the other AMB, warranting us to grow the cells without antibiotics.

Another limitation we encountered was how to quantify cytochrome c release. The discrete nature of the healthy mitochondria was amenable to qualitative description, but not unbiased quantitation. Lessons learned here inspired the acquisition of photocytometric software to resolve the issue of quantifying both healthy and unhealthy mitochondria.

Finally, most of the cell culture work was done in live cells, care should be exercised in interpreting the results because of the simplicity of the system when compared to humans. The complex homeostatic mechanisms in whole animal models are thus not represented in our *in vitro* model. The culture of the cells in a serum-free environment means the pharmacokinetic role of the high plasma lipoproteins and tissue affinity associated with parenteral AMB (but not AME) were circumvented.

Despite the narrative in early medical literature suggesting AME is neurotoxic, accumulating evidence provides clarity that highly purified AME devoid of its parent drug AMB as a contaminant is a safe drug, both in the context of general toxicity and neurotoxicity. This study supports the conclusion that AMB is myelinotoxic by inducing both plasma membrane and mitochondrial membrane permeability in oligodendrocytes with consequent apoptosis, effects which were not seen following clinically relevant concentrations of AME. Access to AME for further scientific and clinical research will be critical to enhancing understanding in the antifungal field, with the potential of preventing needless death and debility.

Chapter 5

5 References

Afifi, N.S., Abdel-Hamid E.S., Baghdadi, H.M., Mohamed,A.F. (2012). Nuclear area factor as an estimate for apoptosis in oral squamous cell carcinoma -treated cell line: A comparative in-vitro study with DNA fragmentation assay. *J. Clinic Experiment Pathology, 2*(107).

Al-Dhaheri, R. S., & Douglas, L. J. (2010). Apoptosis in candida biofilms exposed to amphotericin B. *Journal of Medical Microbiology, 59*(Pt 2), 149-157.

Amstad, P. A., Yu, G., Johnson, G. L., Lee, B. W., Dhawan, S., Phelps, D. J. (2001). Detection of caspase activation in situ by fluorochrome-labeled caspase inhibitors. *BioTechniques, 31*(3), 608-10, 612, 614, passim.

Armstrong-James, D., Meintjes, G., Brown, G.D. (2014) A neglected epidemic: fungal infections in HIV/AIDS., *Trends in Microbiology, 22*,120-127

Arsura, E. L., Johnson, R., Penrose, J., Stewart, K., Kilgore, W., Reddy, C. M. (2005). Neuroimaging as a guide to predict outcomes for patients with coccidioidal meningitis. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *40*(4), 624-627.

Atkinson, A. J.,Jr, & Bennett, J. E. (1978). Amphotericin B pharmacokinetics in humans. *Antimicrobial Agents and Chemotherapy, 13*(2), 271-276.

Baginski, M., Resat, H., & McCammon, J. A. (1997). Molecular properties of amphotericin B membrane channel: A molecular dynamics stimulation. Mol Pharmacol, 52(4), 560-70.

Banuelos, A. F., Williams, P. L., Johnson, R. H., Bibi, S., Fredricks, D. N., Gilroy, S. A., et al. (1996). Central nervous system abscesses due to coccidioides species. *Clinical Infectious Disease: An Official Publication of the Infectious Diseases Society of America, 22*(2), 240-250.

Barnato, A. E., Sanders, G. D., & Owens, D. K. (2001). Cost-effectiveness of a potential vaccine for *Coccidioides immitis*. *Emerging Infectious Diseases, 7*(5), 797-806.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A., & Bernardi, P. (2005). Properties of the permeability transition pore in mitochondria devoid of cyclophilin D. *The Journal of Biological Chemistry, 280*(19), 18558-18561.

Baumann, N., & Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiological Reviews, 81*(2), 871-927.

Bedner, E., Li, X., Kunicki, J., & Darzynkiewicz, Z. (2000). Translocation of bax to mitochondria during apoptosis measured by laser scanning cytometry. *Cytometry, 41*(2), 83-88.

Bedner, E., Smolewski, P., Amstad, P., & Darzynkiewicz, Z. (2000). Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases

(FLICA): Correlation with DNA fragmentation. *Experimental Cell Research, 259*(1), 308-313.

Bekersky, I., Fielding, R. M., Dressler, D. E., Lee, J. W., Buell, D. N., & Walsh, T. J. (2002). Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. *Antimicrobial Agents and Chemotherapy, 46*(3), 828-833.

Bekersky, I., Fielding, R. M., Dressler, D. E., Kline, S., Buell, D. N., & Walsh, T. J. (2001). Pharmacokinetics, excretion, and mass balance of 14C after administration of 14C-cholesterol-labeled AmBisome to healthy volunteers. *Journal of Clinical Pharmacology, 41*(9), 963-971.

Bernardi, P. (1999). Mitochondrial transport of cations: Channels, exchangers, and permeability transition. *Physiological Reviews, 79*(4), 1127-1155.

Bertrand, R., Solary, E., O'Connor, P., Kohn, K. W., & Pommier, Y. (1994). Induction of a common pathway of apoptosis by staurosporine. *Experimental Cell Research, 211*(2), 314-321.

Bolard, J. (1986). How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochimica Et Biophysica Acta, 864*(3-4), 257-304.

Bolard, J., Cheron, M., & Mazerski, J. (1984). Effect of surface curvature on the interaction of single lamellar phospholipid vesicles with aromatic and nonaromatic

heptaene antibiotics (vacidin A and amphotericin B). *Biochemical Pharmacology, 33*(22), 3675-3680.

Bolard, J., Seigneuret, M., & Boudet, G. (1980). Interaction between phospholipid bilayer membranes and the polyene antibiotic amphotericin B: Lipid state and cholesterol content dependence. *Biochimica Et Biophysica Acta, 599*(1), 280-293.

Bonner, D. P., Mechlinski, W., & Schaffner, C. P. (1972). Polyene macrolide derivatives. 3. Biological properties of polyene macrolide ester salts. *The Journal of Antibiotics, 25*(4), 261-262.

Bonner, D. P., Mechlinski, W., & Schaffner, C. P. (1975). Stability studies with amphotericin B and amphotericin B methyl ester. *The Journal of Antibiotics, 28*(2), 132-135.

Bonner, D. P., Tewari, R. P., Solotorovsky, M., Mechlinski, W., & Schaffner, C. P. (1975). Comparative chemotherapeutic activity of amphotericin B and amphotericin B methy ester. *Antimicrobial Agents and Chemotherapy, 7*(6), 724-729.

Bonner, D. P., Fisher, P. B., Goldstein, N. I., Mechlinski, W., Bryson, V., & Schaffner, C. P. (1976). The effect of fetal bovine serum on polyene macrolide antibiotic cytotoxicity and antifungal activity. *In Vitro, 12*(5), 399-404.

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: Biology and pathology. *Acta Neuropathologica, 119*(1), 37-53.

Brajtburg, J., & Bolard, J. (1996). Carrier effects on biological activity of amphotericin B. *Clinical Microbiology Reviews, 9*(4), 512-531.

Brajtburg, J., Powderly, W. G., Kobayashi, G. S., & Medoff, G. (1990). Amphotericin B: Current understanding of mechanisms of action. *Antimicrobial Agents and Chemotherapy, 34*(2), 183-188.

Brookes, P. S., Land, J. M., Clark, J. B., & Heales, S. J. (1998). Peroxynitrite and brain mitochondria: Evidence for increased proton leak. *Journal of Neurochemistry,70*(5), 2195-2202.

Buntinx, M., Vanderlocht, J., Hellings, N., Vandenabeele, F., Lambrichts, I., Raus, J., et al. (2003). Characterization of three human oligodendroglial cell lines as a model to study oligodendrocyte injury: Morphology and oligodendrocyte-specific gene expression. *Journal of Neurocytology, 32*(1), 25-38.

Carnevale, N. T., Galgiani, J. N., Stevens, D. A., Herrick, M. K., & Langston, J. W. (1980). Amphotericin B-induced myelopathy. *Archives of Internal Medicine, 140*(9), 1189-1192.

Cass, A., Finkelstein, A., & Krespi, V. (1970). The ion permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *The Journal of General Physiology, 56*(1), 100-124.

Cesura, A. M., Pinard, E., Schubenel, R., Goetschy, V., Friedlein, A., Langen, H. (2003). The voltage-dependent anion channel is the target for a new class of inhibitors of the

mitochondrial permeability transition pore. *The Journal of Biological Chemistry, 278*(50), 49812-49818.

Chen, W. C., Chou, D. L., & Feingold, D. S. (1978). Dissociation between ion permeability and the lethal action of polyene antibiotics on *Candida albicans*. *Antimicrobial Agents and Chemotherapy, 13*(6), 914-917.

Chen, W. C., Sud, I. J., Chou, D. L., & Feingold, D. S. (1977). Selective toxicity of the polyene antibiotics and their methyl ester derivatives. *Biochemical and Biophysical Research Communications, 74*(2), 480-487.

Cheron, M., Cybulska, B., Mazerski, J., Grzybowska, J., Czerwinski, A., & Borowski, E. (1988). Quantitative structure-activity relationships in amphotericin B derivatives. *Biochemical Pharmacology, 37*(5), 827-836.

Chiller, T. M., Galgiani, J. N., & Stevens, D. A. (2003). Coccidioidomycosis. *Infectious Disease Clinics of North America, 17*(1), 41-57, viii.

Clark, T. A., & Hajjeh, R. A. (2002). Recent trends in the epidemiology of invasive mycoses. *Current Opinion in Infectious Diseases, 15*(6), 569-574.

Cohen, B. E. (1992). A sequential mechanism for the formation of aqueous channels by amphotericin B in liposomes. the effect of sterols and phospholipid composition. *Biochimica Et Biophysica Acta, 1108*(1), 49-58.

Cohen, B. E. (2010). Amphotericin B membrane action: Role for two types of ion channels in eliciting cell survival and lethal effects. *The Journal of Membrane Biology, 238*(1-3), 1-20.

Collette, N., van der Auwera, P., Lopez, A. P., Heymans, C., & Meunier, F. (1989). Tissue concentrations and bioactivity of amphotericin B in cancer patients treated with amphotericin B-deoxycholate. *Antimicrobial Agents and Chemotherapy, 33*(3), 362-368.

Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G., & Franceschi, C. (1993). A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochemical and Biophysical Research Communications, 197*(1), 40-45.

Craighead, M., Pole, J., & Waters, C. (2000). Caspases mediate C2-ceramide-induced apoptosis of the human oligodendroglial cell line, MO3.13. *Neuroscience Letters, 278*(3), 125-128.

Craighead, M. W., Tiwari, P., Keynes, R. G., & Waters, C. M. (1999). Human oligodendroglial cell line, MO3.13, can be protected from apoptosis using the general caspase inhibitor zVAD-FMK. *Journal of Neuroscience Research, 57*(2), 236-243.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *The Biochemical Journal, 34(Pt 2)*(Pt 2), 233-249.

Crum, N. F., Lederman, E. R., Stafford, C. M., Parrish, J. S., & Wallace, M. R. (2004). Coccidioidomycosis: A descriptive survey of a reemerging disease. Clinical characteristics and current controversies. Medicine, 83(3), 149-175.

Cybulska, B., Gadomska, I., Mazerski, J., Borowski, J. G. E., Cheron, M., & Bolard, J. (2000). N-methyl-N-D-fructosyl amphotericin B methyl ester (MF-AME), a novel antifungal agent of low toxicity: Monomer/micelle control over selective toxicity. *Acta Biochimica Polonica, 47*(1), 121-131.

DeCoster, M. (2007). The nuclear area factor (NAF): A measure for cell apoptosis using microscopy and image analysis. *Modern Research and Educational Topics in Microscopy, 1*,378-384.

de Resende, M. A., & Alterthum, F. (1990). Effect of nystatin, amphotericin B and amphotericin B methyl ester on *Saccharomyces cerevisiae* with different lipid composition. *Mycopathologia, 112*(3), 165-172.

Debatin, K. M., Poncet, D., & Kroemer, G. (2002). Chemotherapy: Targeting the mitochondrial cell death pathway. *Oncogene, 21*(57), 8786-8803.

Debono, M., & Gordee, R. S. (1994). Antibiotics that inhibit fungal cell wall development. *Annual Review of Microbiology, 48*, 471-497.

Deresinski, S. (2003). Coccidioides immitis as a potential bioweapon. *Seminars in Respiratory Infections, 18*(3), 216-219.

Devinsky, O., Lemann, W., Evans, A. C., Moeller, J. R., & Rottenberg, D. A. (1987). Akinetic mutism in a bone marrow transplant recipient following total-body irradiation and amphotericin B chemoprophylaxis. A positron emission tomographic and neuropathologic study. *Archives of Neurology, 44*(4), 414-417.

DiCaudo, D. J. (2006).Coccidioidomycosis: A review and update.*Journal of the American Academy of Dermatology, 55*(6), 929-42; quiz 943-5.

DiMasi, J. A. (2001). Risks in new drug development: Approval success rates for investigational drugs. *Clinical Pharmacology and Therapeutics, 69*(5), 297-307.

DiMasi, J. A., Hansen, R. W., & Grabowski, H. G. (2003). The price of innovation: New estimates of drug development costs. *Journal of Health Economics, 22*(2), 151-185.

Dixon, D. M. (2001).Coccidioides immitis as a select agent of bioterrorism. *Journal of Applied Microbiology, 91*(4), 602-605.

Dutcher, J. D. (1968). The discovery and development of amphotericin B. *Diseases of the Chest, 54*, Suppl 1:296-8.

Eccleston, P. A., & Silberberg, D. H. (1984). The differentiation of oligodendrocytes in a serum-free hormone-supplemented medium. *Brain Research, 318*(1), 1-9.

Edgar, J. M., & Garbern, J. (2004). The myelinated axon is dependent on the myelinating cell for support and maintenance: Molecules involved. *Journal of Neuroscience Research, 76*(5), 593-598.

Einstein, H. E., Holeman, C. W.,Jr, Sandidge, L. L., & Holden, D. H. (1961). Coccidioidal meningitis. The use of amphotericin B in treatment. *California Medicine, 94*, 339-343.

ElAli, A., Theriault, P., & Rivest, S. (2014). The role of pericytes in neurovascular unit remodeling in brain disorders. *International Journal of Molecular Sciences, 15*(4), 6453-6474.

Ellis, W. G., Bencken, E., LeCouteur, R. A., Barbano, J. R., Wolfe, B. M., & Jennings, M. B. (1988). Neurotoxicity of amphotericin B methyl ester in dogs. *Toxicologic Pathology, 16*(1), 1-9.

Ellis, W. G., Sobel, R. A., & Nielsen, S. L. (1982). Leukoencephalopathy in patients treated with amphotericin B methyl ester. *The Journal of Infectious Diseases, 146*(2), 125-137.

Espinel-Ingroff, A. (1996). History of medical mycology in the United States. *Clinical Microbiology Reviews, 9*(2), 235-272.

Espinel-Ingroff, A., & Shadomy, S. (1989). In vitro and in vivo evaluation of antifungal agents. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 8*(4), 352-361.

Fanos, V., & Cataldi, L. (2000). Amphotericin B-induced nephrotoxicity: A review. *Journal of Chemotherapy (Florence, Italy), 12*(6), 463-470.

Fisher, J. F., & Dewald, J. (1983). Parkinsonism associated with intraventricular amphotericin B. *The Journal of Antimicrobial Chemotherapy, 12*(1), 97-99.

Fisher, P. B., Bryson, V., & Schaffner, C. P. (1978). Polyene macrolide antibiotic cytotoxicity and membrane permeability alterations. I. Comparative effects of four classes of polyene macrolides on mammalian cells. *Journal of Cellular Physiology, 97*(3 Pt 1), 345-351.

Fisher, P. B., Bryson, V., & Schaffner, C. P. (1979). Polyene macrolide antibiotic cytotoxicity and membrane permeability alterations. II. Phenotypic expression in intraspecific and interspecific somatic cell hybrids. *Journal of Cellular Physiology, 100*(2), 335-342.

Fisher, P. B., Goldstein, N. I., Bonner, D. P., Mechlinski, W., Bryson, V., & Schaffner, C. P. (1975). Stimulatory effect of amphotericin B methyl ester on the growth of L-M and vero cells. *The Journal of Antibiotics, 28*(11), 896-902.

Fisher, P. B., Goldstein, N. I., Bonner, D. P., Mechlinski, W., Bryson, V., & Schaffner, C. P. (1975). Toxicity of amphotericin B and its methyl ester toward normal and tumor cell lines. *Cancer Research, 35*(8), 1996-1999.

Fisher, P. B., Goldstein, N. I., Bryson, V., & Schaffner, C. P. (1976). Reduced toxicity of amphotericin B methyl ester (AME) vs. amphotericin B and Fungizone in tissue culture. *In Vitro, 12*(2), 133-140.

Gadebusch, H. H., Pansy, F., Klepner, C., & Schwind, R. (1976). Amphotericin B and amphotericin B methyl ester ascorbate. I. Chemotherapeutic activity against *Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis* in mice. *The Journal of Infectious Diseases, 134*(5), 423-427.

Gale, E. F. (1974). The release of potassium ions from candida albicans in the presence of polyene antibiotics. *Journal of General Microbiology, 80*(2), 451-465.

Galgiani, J. N., Ampel, N. M., Catanzaro, A., Johnson, R. H., Stevens, D. A., & Williams, P. L. (2000). Practice guideline for the treatment of coccidioidomycosis. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 30*(4), 658-661.

Galgiani, J. N., Ampel, N. M., Blair, J. E., Catanzaro, A., Johnson, R. H., Stevens, D. A., et al. (2005). Coccidioidomycosis. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 41*(9), 1217-1223.

Georgopapadakou, N. H., & Walsh, T. J. (1994). Human mycoses: Drugs and targets for emerging pathogens. *Science (New York, N.Y.), 264*(5157), 371-373.

Gold, B.G., Ryzlak, M.T., Schaffner, C.P. (1989). Comparison of the neuropathologies of amphotericin b (AB) and its methyl ester derivative (AME) in peripheral nerve. *Society for Neuroscience Abstracts*. *15*(2) 1348.

Gold, B.G., Ryzlak, M.T., Schaffner, C.P. (1990). Primary and secondary demyelination produced by injection of amphotericin b (AB) or its methyl ester analogue (AME) into the rat sciatic nerve. *Journal of Neuropathology & Experimental Neurology*, *49*(292)

Goldstein, J. C., Kluck, R. M., & Green, D. R. (2000). A single cell analysis of apoptosis. Ordering the apoptotic phenotype. *Annals of the New York Academy of Sciences, 926*, 132-141.

Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., & Green, D. R. (2000). The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nature Cell Biology, 2*(3), 156-162.

Graybill, J. R., Stevens, D. A., Galgiani, J. N., Sugar, A. M., Craven, P. C., Gregg, C., et al. (1988). Ketoconazole treatment of coccidioidal meningitis. *Annals of the New York Academy of Sciences*, *544*, 488-496.

Groll, A. H., Mickiene, D., Piscitelli, S. C., & Walsh, T. J. (2000). Distribution of lipid formulations of amphotericin B into bone marrow and fat tissue in rabbits. *Antimicrobial Agents and Chemotherapy, 44*(2), 408-410.

PO Gubbins, EJ Anaissie –(2009) Clinical mycology, 2nd edn. Elsevier, - books.google.com

Haber, R. W., & Joseph, M. (1962). Neurological manifestations after amphotericin B therapy. *British Medical Journal, 1*(5273), 230-231.

Halestrap, A. P., & Brenner, C. (2003). The adenine nucleotide translocase: A central component of the mitochondrial permeability transition pore and key player in cell death. *Current Medicinal Chemistry, 10*(16), 1507-1525.

Halestrap, A. P., McStay, G. P., & Clarke, S. J. (2002). The permeability transition pore complex: Another view. *Biochimie, 84*(2-3), 153-166.

Hamdan, J. S., & de Resende, M. A. (1988). Lipid composition and effect of amphotericin B on yeast cells of *Paracoccidioides brasiliensis*. *Mycopathologia, 102*(2), 97-105.

Hamilton-Miller, J. M. (1973). Chemistry and biology of the polyene macrolide antibiotics. *Bacteriological Reviews, 37*(3), 166-196.

Hamilton-Miller, J. M. (1974). Fungal sterols and the mode of action of the polyene antibiotics. *Advances in Applied Microbiology, 17*(0), 109-134.

Hammond, S. M. (1977). Biological activity of polyene antibiotics. *Progress in Medicinal Chemistry, 14*, 105-179.

Hammond, S. M., Lambert, P. A., & Kliger, B. N. (1974). The mode of action of polyene antibiotics; induced potassium leakage in *Candida albicans*. *Journal of General Microbiology, 81*(2), 325-330.

Haq, E., Giri, S., Singh, I., & Singh, A. K. (2003). Molecular mechanism of psychosine-induced cell death in human oligodendrocyte cell line. *Journal of Neurochemistry, 86*(6), 1428-1440.

Hartsel, S., & Bolard, J. (1996). Amphotericin B: New life for an old drug. *Trends in Pharmacological Sciences, 17*(12), 445-449.

Haworth, R. A., & Hunter, D. R. (1979). The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. *Archives of Biochemistry and Biophysics, 195*(2), 460-467.

Henderson, P. J., & Lardy, H. A. (1970). Bongkrekic acid an inhibitor of the adenine nucleotide translocase of mitochondria. *The Journal of Biological Chemistry, 245*(6), 1319-1326.

Hoeprich, P. (1982). Amphotericin B methyl ester and leukoencephalopathy: The other side of the coin. *The Journal of Infectious Diseases, 146*(2), 173-176.

Hoeprich, P. D. (1989). Chemotherapy for systemic mycoses. *Progress in Drug Research.Fortschritte Der Arzneimittelforschung.Progres Des Recherches Pharmaceutiques, 33*, 317-351.

Hoeprich, P. D. (1992). Clinical use of amphotericin B and derivatives: Lore, mystique, and fact. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 14 Suppl 1*, S114-9.

Hoeprich, P. D., Flynn, N. M., Kawachi, M. M., Lee, K. K., Lawrence, R. M., Heath, L. K., et al. (1988). Treatment of fungal infections with semisynthetic derivatives of amphotericin B alpha. *Annals of the New York Academy of Sciences, 544*, 517-546.

Hoeprich, P. D., & Huston, A. C. (1976). Effect of culture media on the antifungal activity of miconazole and amphotericin B methyl ester. *The Journal of Infectious Diseases, 134*(4), 336-341.

Hoeprich, P. D., Huston, A. C., & Wolfe, B. M. (1985). Toxicity of amphotericins on chronic administration to mongrel dogs. *Diagnostic Microbiology and Infectious Disease, 3*(1), 47-58.

Hoeprich, P. D., Merry, J. M., Gunther, R., & Franti, C. E. (1987). Entry of five antifungal agents into the ovine lung. *Antimicrobial Agents and Chemotherapy, 31*(8), 1234-1237.

Howarth, W. R., Tewari, R. P., & Solotorovsky, M. (1975). Comparative in vitro antifungal activity of amphotericin B and amphotericin B methyl ester. *Antimicrobial Agents and Chemotherapy, 7*(1), 58-63.

Hughes, F. M.,Jr, & Cidlowski, J. A. (1997). Utilization of an in vitro assay to evaluate chromatin degradation by candidate apoptotic nucleases. *Cell Death and Differentiation, 4*(3), 200-208.

Hunter, D. R., & Haworth, R. A. (1979). The Ca2+-induced membrane transition in mitochondria. III. Transitional Ca2+ release. *Archives of Biochemistry and Biophysics, 195*(2), 468-477.

Huston, A. C., & Hoeprich, P. D. (1978). Comparative susceptibility of four kinds of pathogenic fungi to amphotericin B and amphotericin B methyl ester. *Antimicrobial Agents and Chemotherapy, 13*(6), 905-909.

Irwin, S. (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia*, 13(3): p. 222-57

Issa, Y., Watts, D. C., Duxbury, A. J., Brunton, P. A., Watson, M. B., & Waters, C. M. (2003). Mercuric chloride: Toxicity and apoptosis in a human oligodendroglial cell line MO3.13. *Biomaterials, 24*(6), 981-987.

Jacotot, E., Costantini, P., Laboureau, E., Zamzami, N., Susin, S. A., & Kroemer, G. (1999). Mitochondrial membrane permeabilization during the apoptotic process. *Annals of the New York Academy of Sciences, 887*, 18-30.

Jagdis, F. A., Hoeprich, P. D., Lawrence, R. M., & Schaffner, C. P. (1977). Comparative pharmacology of amphotericin B and amphotericin B methyl ester in the non-human primate, *Macacca mulatta*. *Antimicrobial Agents and Chemotherapy, 12*(5), 582-590

Janknegt, R., de Marie, S., Bakker-Woudenberg, I. A., & Crommelin, D. J. (1992). Liposomal and lipid formulations of amphotericin B. *Clinical Pharmacokinetics, 23*(4), 279-291.

Johnson, R. H., & Einstein, H. E. (2006). Coccidioidal meningitis. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 42*(1), 103-107.

Juurlink, B. H. (1997). Response of glial cells to ischemia: Roles of reactive oxygen species and glutathione. *Neuroscience and Biobehavioral Reviews, 21*(2), 151-166.

Kanaan, A., Farahani, R., Douglas, R. M., Lamanna, J. C., & Haddad, G. G. (2006). Effect of chronic continuous or intermittent hypoxia and reoxygenation on cerebral capillary density and myelination. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 290*(4), R1105-14.

Katzman, R., Aleu, F., & Wilson, C. (1963). Further observations on triethyltin edema. *Archives of Neurology, 9*, 178-187.

Keim, G. R.,Jr, Poutsiaka, J. W., Kirpan, J., & Keysser, C. H. (1973). Amphotericin B methyl ester hydrochloride and amphotericin B: Comparative acute toxicity. *Science (New York, N.Y.), 179*(73), 584-585.

Keim, G. R., Sibley, P. L., Yoon, Y. H., Kulesza, J. S., Zaidi, I. H., Miller, M. M., et al. (1976). Comparative toxicological studies of amphotericin B methyl ester and amphotericin B in mice, rats, and dogs. *Antimicrobial Agents and Chemotherapy, 10*(4), 687-690.

Kethireddy, S., & Andes, D. (2007). CNS pharmacokinetics of antifungal agents. *Expert Opinion on Drug Metabolism & Toxicology, 3*(4), 573-581.

Kinsky, S. C., Avruch, J., Permutt, M., & Rogers, H. B. (1962). The lytic effect of polyene antifungal antibiotics on mammalian erythrocytes. *Biochemical and Biophysical Research Communications, 9*, 503-507.

Kinsky, S. C., Luse, S. A., & van Deenen, L. L. (1966). Interaction of polyene antibiotics with natural and artificial membrane systems. *Federation Proceedings, 25*(5), 1503-1510.

Kintzel, P. E., & Kennedy, P. E. (1991). Stability of amphotericin B in 5% dextrose injection at 25 degrees C. *American Journal of Hospital Pharmacy, 48*(8), 1681.

Kintzel, P. E., & Kennedy, P. E. (1991). Stability of amphotericin B in 5% dextrose injection at concentrations used for administration through a central venous line. *American Journal of Hospital Pharmacy, 48*(2), 283-285.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: A primary site for bcl-2 regulation of apoptosis. *Science (New York, N.Y.), 275*(5303), 1132-1136.

Koldin, M. H., Kobayashi, G. S., Brajtburg, J., & Medoff, G. (1985). Effects of elevation of serum cholesterol and administration of amphotericin B complexed to lipoproteins on amphotericin B-induced toxicity in rabbits. *Antimicrobial Agents and Chemotherapy, 28*(1), 144-145.

Kozmin, S. A. (2008). Chemical "knockout" challenges the amphotericin B channel model. *Nature Chemical Biology, 4*(1), 19-20.

Lampen, J. O. (1969). Amphotericin B and other polyenic antifungal antibiotics. *American Journal of Clinical Pathology, 52*(2), 138-146

Lawrence, R. M., & Hoeprich, P. D. (1976). Comparison of amphotericin B and amphotericin B methyl ester: Efficacy in murine coccidioidomycosis and toxicity. *The Journal of Infectious Diseases, 133*(2), 168-174.

Lawrence, R. M., Hoeprich, P. D., Jagdis, F. A., Monji, N., Huston, A. C., & Schaffner, C. P. (1980). Distribution of doubly radiolabelled amphotericin B methyl ester and amphotericin B in the non-human primate, *Macaca mulatta*. *The Journal of Antimicrobial Chemotherapy, 6*(2), 241-249.

Lee, M. D., Hess, M. M., Boucher, B. A., & Apple, A. M. (1994). Stability of amphotericin B in 5% dextrose injection stored at 4 or 25 degrees C for 120 hours. *American Journal of Hospital Pharmacy, 51*(3), 394-396.

Lemke, A., Kiderlen, A.F., Kayser, O. (2005). Amphotericin B. *Appl Microbiol Biotechnol*., *68*(2):151-62

Lewis, R. E., & Wiederhold, N. P. (2003). The solubility ceiling: A rationale for continuous infusion amphotericin B therapy? *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 37*(6), 871-872.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., et al. (1997). Cytochrome c and dATP-dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91*(4), 479-489.

Li, P. K., & Lai, K. N. (1989). Amphotericin B induced ocular toxicity in cryptococcal meningitis. *The British Journal of Ophthalmology, 73*(5), 397-398.

Lieven, C. J., Vrabec, J. P., & Levin, L. A. (2003). The effects of oxidative stress on mitochondrial transmembrane potential in retinal ganglion cells. Antioxidants & Redox Signaling, 5(5), 641-646.

Linke, H. A., Mechlinski, W., & Schaffner, C. P. (1974). Production of amphotericin B-14C by *Streptomyces nodosus* fermentation, and preparation of the amphotericin B-14C-methyl-ester. *The Journal of Antibiotics, 27*(3), 155-160.

Liu, J. S., Chang, Y. Y., Chen, W. H., & Chen, S. S. (1995). Amphotericin B-induced leukoencephalopathy in a patient with cryptococcal meningitis. *Journal of the Formosan Medical Association = Taiwan Yi Zhi, 94*(7), 432-434.

Marklund, L., Behnam-Motlagh, P., Henriksson, R., & Grankvist, K. (2001). Bumetanide annihilation of amphotericin B-induced apoptosis and cytotoxicity is due to its effect on cellular K+ flux. *The Journal of Antimicrobial Chemotherapy, 48*(6), 781-786.

Massa, T., Sinha, D. P., Frantz, J. D., Filipek, M. E., Weglein, R. C., Steinberg, S. A., et al. (1985). Subchronic toxicity studies of N-D-ornithyl amphotericin B methyl ester in dogs and rats. *Fundamental and Applied Toxicology : Official Journal of the Society of Toxicology, 5*(4), 737-753.

Mathisen, G., Shelub, A., Truong, J., & Wigen, C. (2010). Coccidioidal meningitis: Clinical presentation and management in the fluconazole era. *Medicine, 89*(5), 251-284.

Mazerski, J., Grzybowska, J., & Borowski, E. (1990). Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media. *European Biophysics Journal: EBJ, 18*(3), 159-164.

McLaurin, J., Trudel, G. C., Shaw, I. T., Antel, J. P., & Cashman, N. R. (1995). A human glial hybrid cell line differentially expressing genes subserving oligodendrocyte and astrocyte phenotype. *Journal of Neurobiology, 26*(2), 283-293.

McLaurin, J. A., & Yong, V. W. (1995). Oligodendrocytes and myelin. *Neurologic Clinics, 13*(1), 23-49.

Mechlinski, W., & Schaffner, C. P. (1972). Polyene macrolide derivatives. I. N-acylation and esterification reactions with amphotericin B. *The Journal of Antibiotics, 25*(4), 256-258.

Mechlinski, W., & Schaffner, C. P. (1974). Separation of polyene antifungal antibiotics by high-speed liquid chromatography. *Journal of Chromatography, 99*(0), 619-633.

Medoff, G., Brajtburg, J., Kobayashi, G. S., & Bolard, J. (1983). Antifungal agents useful in therapy of systemic fungal infections. *Annual Review of Pharmacology and Toxicology, 23*, 303-330.

Monji, N., Bonner, D. P., Hashimoto, Y., & Schaffner, C. P. (1975). Studies on the absorption, distribution and excretion of radioactivity after intravenous and intraperitoneal administration of 14C-methyl ester of amphotericin B. *The Journal of Antibiotics, 28*(4), 317-324.

Moreira, W., Leprohon, P., & Ouellette, M. (2011). Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in leishmania. *Cell Death & Disease, 2*, e201.

Morell, P., & Toews, A. D. (1996). Schwann cells as targets for neurotoxicants. *Neurotoxicology, 17*(3-4), 685-695.

Motoyoshi, A., Nakajima, H., Takano, K., Moriyama, M., Kannan, Y., & Nakamura, Y. (2008). Effects of amphotericin B on the expression of neurotoxic and neurotrophic factors in cultured microglia. Neurochemistry International, 52(6), 1290-1296.

Mousavi, S. A., & Robson, G. D. (2004). Oxidative and amphotericin B-mediated cell death in the opportunistic pathogen *Aspergillus fumigatus* is associated with an apoptotic-like phenotype. *Microbiology (Reading, England), 150*(Pt 6), 1937-1945.

Nicholson, D. W., & Thornberry, N. A. (1997). Caspases: Killer proteases. Trends in Biochemical Sciences, 22(8), 299-306.

Neihart, R. E., Hinthorn, D. R., Hoeprich, P. D., & Liu, C. (1989). Successful treatment of progressive disseminated histoplasmosis with amphotericin B methyl ester.Diagnostic Microbiology and Infectious Disease, 12(1), 17-19.

Oblack, D. L., Hewitt, W. L., & Martin, W. J. (1981). Comparative in vitro susceptibility of yeasts to amphotericin B and three methyl ester derivatives. *Antimicrobial Agents and Chemotherapy, 19*(1), 106-109.

Odabasi, Z., Karaalp, A., Cermik, H., Mohr, J., Tigen, E. T., Koc, M., et al. (2009). Reduction of amphotericin B-induced renal tubular apoptosis by N-acetylcysteine. *Antimicrobial Agents and Chemotherapy, 53*(7), 3100-3102.

Oldfield, E. C., Bone, W. D., Martin, C. R., Gray, G. C., Olson, P., & Schillaci, R. F. (1997). Prediction of relapse after treatment of coccidioidomycosis. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 25(5), 1205-1210.

Palacios, D. S., Anderson, T. M., & Burke, M. D. (2007). A post-PKS oxidation of the amphotericin B skeleton predicted to be critical for channel formation is not required for potent antifungal activity. *Journal of the American Chemical Society, 129*(45), 13804-13805.

Phillips, A. J., Sudbery, I., &Ramsdale, M. (2003). Apoptosis induced by environmental stresses and amphotericin B in C*andida albicans*. *Proceedings of the National Academy of Sciences of the United States of America, 100*(24), 14327-14332.

Racis, S. P., Plescia, O. J., Geller, H. M., & Schaffner, C. P. (1990). Comparative toxicities of amphotericin B and its monomethyl ester derivative on glial cells in culture. *Antimicrobial Agents and Chemotherapy, 34*(7), 1360-1365.

Reuhl, K. R., Vapiwala, M., Ryzlak, M. T., & Schaffner, C. P. (1993). Comparative neurotoxicities of amphotericin B and its mono-methyl ester derivative in rats. *Antimicrobial Agents and Chemotherapy, 37*(3), 419-428.

Richardson, M. D. (2005). Changing patterns and trends in systemic fungal infections. *Journal of Antimicrobial Chemotherapy, 56*(suppl 1), i5-i11.

Rinehart, K. L.,Jr. (1982). Fast atom bombardment mass spectrometry. *Science (New York, N.Y.), 218*(4569), 254-260.

Ritter, J. M., Muehlenbachs, A., Blau, D. M., Paddock, C. D., Shieh, W. J., Drew, C. P., et al. (2013). Exserohilum infections associated with contaminated steroid injections: A clinicopathologic review of 40 cases. *The American Journal of Pathology, 183*(3), 881-892.

Romanini, D., Avalle, G., Nerli, B., & Pico, G. (1999). Thermodynamic and spectroscopic features of the behavior of amphotericin B in aqueous medium. *Biophysical Chemistry, 77*(1), 69-77.

Sabra, R., & Branch, R. A. (1990). Amphotericin B nephrotoxicity. *Drug Safety : An International Journal of Medical Toxicology and Drug Experience, 5*(2), 94-108.

Sangamwar, A. T., Deshpande, U. D., & Pekamwar, S. S. (2008). Antifungals: Need to search for a new molecular target. *Indian Journal of Pharmaceutical Sciences, 70*(4), 423-430.

Sawaya, B. P., Weihprecht, H., Campbell, W. R., Lorenz, J. N., Webb, R. C., Briggs, J. P., et al. (1991). Direct vasoconstriction as a possible cause for amphotericin B-induced nephrotoxicity in rats. *The Journal of Clinical Investigation, 87*(6), 2097-2107.

Schaffner, C. P., & Mechlinski, W. (1972). Polyene macrolide derivatives. II. Physical-chemical properties of polyene macrolide esters and their water soluble salts. *The Journal of Antibiotics, 25*(4), 259-260.

Schell, R. E. (1993). Proton permeability of renal membranes: Influence of amphotericin B. Nephron, 63(4), 481.

Shadomy, S., Brummer, D. L., & Ingroff, A. V. (1973). Light sensitivity of prepared solutions of amphotericin B. *The American Review of Respiratory Disease, 107*(2), 303-304.

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., et al. (1999). Ordering the cytochrome c-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *The Journal of Cell Biology, 144*(2), 281-292.

Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., et al. (1991). Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences of the United States of America, 88*(9), 3671-3675.

Smith, C. E., & Beard, R. R. (1946). Effect of season and dust control on coccidioidomycosis. *Journal of the American Medical Association, 132*(14), 833-838.

Smolewski, P., Bedner, E., Du, L., Hsieh, T. C., Wu, J. M., Phelps, D. J., et al. (2001). Detection of caspases activation by fluorochrome-labeled inhibitors: Multiparameter analysis by laser scanning cytometry. *Cytometry, 44*(1), 73-82.

Sobel, R. A., Ellis, W. G., Nielsen, S. L., & Davis, R. L. (1984). Central nervous system coccidioidomycosis: A clinicopathologic study of treatment with and without amphotericin B. *Human Pathology, 15*(10), 980-995.

Stahnke, T., & Richter-Landsberg, C. (2004). Triethyltin-induced stress responses and apoptotic cell death in cultured oligodendrocytes. *Glia, 46*(3), 334-344.

Stevens, D. A. (1995). Coccidioidomycosis. *The New England Journal of Medicine, 332*(16), 1077-1082.

Stevens, N. M., Engle, C. G., Fisher, P. B., Mechlinski, W., & Schaffner, C. P. (1975). In vitro antiherpetic activity of water-soluble amphotericin B methyl ester. *Archives of Virology, 48*(4), 391-394.

Stevens, D. A., Rendon, A., Gaona-Flores, V., Catanzaro, A., Anstead, G. M., Pedicone, L., et al. (2007). Posaconazole therapy for chronic refractory coccidioidomycosis. *Chest*, 132(3), 952-958.

Stiller, E. T., Vandeputte, J., & Wachtel, J. L. (1955). Amphotericins A and B, antifungal antibiotics produced by a streptomycete. II. The isolation and properties of the crystalline amphotericins. *Antibiot Annu., 3*, 587-91.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., et al. (1999). Mitochondrial release of caspase-2 and -9 during the apoptotic process. *The Journal of Experimental Medicine, 189*(2), 381-394.

Szlinder-Richert, J., Cybulska, B., Grzybowska, J., Borowski, E., & Prasad, R. (2000). Comparative studies on cell stimulatory, permeabilizing and toxic effects induced in sensitive and multidrug resistant fungal strains by amphotericin B (AMB) and N-methyl-N-D-fructosyl amphotericin B methyl ester (MFAME). *ActaBiochimicaPolonica, 47*(1), 133-140.

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., & Martin, G. R. (1979). Laminin--a glycoprotein from basement membranes. *The Journal of Biological Chemistry, 254*(19), 9933-9937.

Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: Enemies within. *Science (New York, N.Y.), 281*(5381), 1312-1316.

Trajkovic, V., Markovic, M., Samardzic, T., Miljkovic, D. J., Popadic, D., & Mostarica Stojkovic, M. (2001). Amphotericin B potentiates the activation of inducible nitric oxide synthase and causes nitric oxide-dependent mitochondrial dysfunction in cytokine-treated rodent astrocytes. *Glia, 35*(3), 180-188.

Utz, J. P. (1980). Chemotherapy for the systemic mycoses: The prelude to ketoconazole. *Reviews of Infectious Diseases, 2*(4), 625-632.

Utz, J. P. (1964). Amphotericin B toxicity; general side effects. *Annals of Internal Medicine, 61*, 340-343.

van Hoogevest, P., & de Kruijff, B. (1978). Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didocosenoyl phosphatidylcholine. A refinement of the model for the formation of pores by amphotericin B in membranes. *Biochimica Et Biophysica Acta, 511*(3), 397-407.

Varlam, D. E., Siddiq, M. M., Parton, L. A., & Russmann, H. (2001). Apoptosis contributes to amphotericin B-induced nephrotoxicity. *Antimicrobial Agents and Chemotherapy, 45*(3), 679-685.

Vincent, T., Galgiani, J. N., Huppert, M., & Salkin, D. (1993). The natural history of coccidioidal meningitis: VA-armed forces cooperative studies, 1955-1958. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 16*(2), 247-254.

Volmer, A. A., & Carreira, E. M. (2010). Active amphotericin B derivatives position the mycosamine in two radial orientations. *Chembiochem: A European Journal of Chemical Biology, 11*(6), 778-781.

Von Ahsen, O., Waterhouse, N. J., Kuwana, T., Newmeyer, D. D., & Green, D. R. (2000). The 'harmless' release of cytochrome c. *Cell Death and Differentiation, 7*(12), 1192-1199.

Waheed, A. A., Ablan, S. D., Mankowski, M. K., Cummins, J. E., Ptak, R. G., Schaffner, C. P., et al. (2006). Inhibition of HIV-1 replication by amphotericin B methyl ester: Selection for resistant variants. *The Journal of Biological Chemistry, 281*(39), 28699-28711.

Waheed, A. A., Ablan, S. D., Soheilian, F., Nagashima, K., Ono, A., Schaffner, C. P., et al. (2008). Inhibition of human immunodeficiency virus type 1 assembly and release by the cholesterol-binding compound amphotericin B methyl ester: Evidence for vpu dependence. *Journal of Virology, 82*(19), 9776-9781.

Waksman, S. A., Lechevalier, H. A., & Schaffner, C. P. (1965). Candicidin and other polyenic antifungal antibiotics. *Bulletin of the World Health Organization, 33*(2), 219-226.

Walker, R. W., & Rosenblum, M. K. (1992). Amphotericin B-associated leukoencephalopathy. *Neurology, 42*(10), 2005-2010.

Warnock, D. W. (2007). Trends in the epidemiology of invasive fungal infections. *Nihon Ishinkin Gakkai Zasshi = Japanese Journal of Medical Mycology, 48*(1), 1-12.

Weddington, W. W., Jr. (1982). Delirium and depression associated with amphotericin B. *Psychosomatics, 23*(10), 1076-1078.

Winn, R. E., Bower, J. H., & Richards, J. F. (1979). Acute toxic delirium. neurotoxicity of intrathecal administration of amphotericin B. *Archives of Internal Medicine, 139*(6), 706-707.

Winn, W. A. (1963). Coccidioidomycosis and amphotericin B. *The Medical Clinics of North America, 47*, 1131-1148.

Wolf, B. B., Schuler, M., Echeverri, F., & Green, D. R. (1999). Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *The Journal of Biological Chemistry, 274*(43), 30651-30656.

Wood, P. M., & Bunge, R. P. (1991). The origin of remyelinating cells in the adult central nervous system: The role of the mature oligodendrocyte. *Glia, 4*(2), 225-232.

Wood, P. M., & Mora, J. (1993). Source of remyelinating oligodendrocytes. *Advances in Neurology, 59*, 113-123. .

Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., et al. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell, 94*(6), 739-750.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., & Wang, X. (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell, 90*(3), 405-413.

Zumbuehl, A., Jeannerat, D., Martin, S. E., Sohrmann, M., Stano, P., Vigassy, T., et al. (2004). An amphotericin B-fluorescein conjugate as a powerful probe for biochemical studies of the membrane. *Angewandte Chemie (International Ed.in English), 43*(39), 5181-5185.

Zumbuehl, A., Stano, P., Heer, D., Walde, P., & Carreira, E. M. (2004). Amphotericin B as a potential probe of the physical state of vesicle membranes. *Organic Letters, 6*(21), 3683-3686.