INTRANASAL DELIVERY FOR PEPTIDE AND SIRNA TO THE BRAIN USING LIPID-BASED NANOCARRIERS FOR THE TREATMENT OF NEUROINFLAMMATION

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SUMMARY

Neurodegenerative diseases are the most prevalent brain diseases affecting more than 5.5 million people worldwide. If left unconstrained, 30 years from now, more than 12 million Americans will suffer from these diseases. Neuro-inflammation has been one of the common denominator in broad spectrum of neurodegenerative diseases, such as Alzheimer’s, Parkinson’s, Sclerosis, and age-related macular degeneration diseases. Prevention and cure of these brain diseases has been facing outstanding challenge due the limited delivery of pharmacological therapeutics to the brain primarily due to presence of blood-brain barrier (BBB), blood-CSF barrier and expression of efflux transporters.

The objective of this thesis project is to develop and characterize a nanoemulsion delivery system to explore the potential of intranasal delivery of cyclosporine peptide and TNF-alpha gene silencing siRNA - based drugs to block the activity of cytokines in LPS-induced animal model of neuro-inflammation. Targeting brain regions by exploiting transport of anti-inflammatory drugs through the olfactory pathways, thus bypassing the BBB, while avoiding peripheral distribution of the drugs and blocking proinflammatory cytokines seems to be a rational approach for the treatment of neuro-inflammation.

We prepared and characterized nanoemulsion formulations for both of the therapeutic drugs: cyclosporine and TNF-alpha silencing siRNA. Multiple cell culture models were screened to mimic the structural and functional aspects of nasal epithelial. RPMI2650 cells demonstrated an enhanced permeability and intracellular uptake of nanoemulsion formulations. With the use of in vivo magnetic resonance imaging (MRI) we qualitatively showed better and higher distribution of nanoemulsion formulations via intranasal dosing. LPS induced neuro-
inflammation based model was established and the anti-inflammatory therapeutic effect and safety of both CSA and TNF-alpha silencing siRNA was proven based on intranasal delivery of nanoemulsion formulation.

We demonstrate that intranasal drug delivery using nanoemulsion systems not only enhances the uptake and distribution in brain but also shows anti-inflammatory therapeutic effects as evidence by reduction in the proinflammatory cytokines in LPS induced neuro-inflammation model. Overall, this study reveals utility of intranasal delivery of nanoemulsion as a non-invasive means to bypass BBB and provides preliminary evidence for a safe and effective drug delivery strategy for targeting brain. These findings open up an opportunity to target at variety of diseases underpinned by inflammation within the nervous system, where exposure of drugs is a major limitation.
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OBJECTIVE AND SPECIFIC AIMS

Prevention and treatment of brain diseases is a major problem due to the limited delivery of pharmacological therapeutics to the brain, primarily due to the blood-brain-barrier (BBB). Intranasal delivery is becoming increasingly important as a route for targeting a range of small and large therapeutic molecules to the central nervous system (CNS) by bypassing the BBB; such delivery limits systemic absorption as well as potential peripheral side effects of administered drugs. A number of investigators have studied intranasal transport of peptide/protein-based drugs for the treatment of CNS disorders, but we have little understanding of how drug delivery systems can be leveraged to enhance nasal residence of the molecules, and how pharmacokinetic profiles of drugs can be altered to afford greater CNS delivery – as a result, many biological molecules are unable to reach the therapeutic levels in the CNS needed for the rapid and sustained action.

The primary objective of this thesis project is to develop and characterize a nanoemulsion delivery system to explore the potential of intranasal delivery of cyclosporine peptide and TNF-alpha/siRNA-based drugs to block the activity of cytokines in LPS-induced animal model of neuro-inflammation. Our working hypotheses for the proposed research are:

1) Encapsulation of therapeutic molecules (peptides or siRNA) into different delivery systems will alter the transport of the drugs, and will affect their mucosal absorption by protecting the biomolecules from proteolytic enzymes, improving their lipophilicity, enhancing their cellular uptake, and increasing their residence time

2) Drug delivery via the nasal route will result in improved CNS uptake and distribution of the drug
3) Targeting of the olfactory epithelium as a route to reach the CNS will reduce the systemic exposure of the drugs, thus limiting their side effects.

The specific aims of the project are as follows:

**Aim 1:** To develop, characterize, and optimize oil-in-water nanoemulsion formulations for intranasal delivery of therapeutic agents: cyclosporine (CSA) and siRNA duplexes

**Aim 2:** To evaluate cellular transport, uptake and cytotoxicity of the delivery systems using nasal epithelial cells

**Aim 3:** To evaluate anti-inflammatory therapeutic response of CSA nanoemulsion (NE) and siRNA NE in LPS-stimulated macrophage in vitro model

**Aim 4:** To qualitatively study the uptake of nanoemulsion in rat brain via intranasal route using magnetic resonance imaging (MRI)

**Aim 5:** To quantitatively compare brain and blood biodistribution and pharmacokinetics of CSA and siRNA delivered intranasally as solution and NE formulation

**Aim 6:** Establish and characterize an LPS-induced neuro-inflammation rat model and examine the therapeutic efficacy of intranasally delivered formulations

**Aim 7:** Assess the acute safety of intranasally delivered formulation in rats
CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Delivery barriers to biological therapies to the CNS

The incidence of brain disorders and neurodegenerative disease is increasing exponentially and treatment of these diseases is found to be extremely challenging due to the blood brain-barrier’s ability to limit entry of all but small, non-polar compounds. Several biotherapeutics including monoclonal antibodies, peptides, proteins, nucleic acids, etc. have shown substantial promise in treating certain aspects of neurodegenerative disease (Alzheimer’s Disease, Parkinson disease, Traumatic brain injury), pain, psychiatric disorders, gliomas diseases. Although biologics have particular advantages over the classical small molecules, because bio-therapeutics is very specific, potent, and have reduced side-effects, delivery of these molecules has been only been possible either through intraparenchymal, intracerebroventricular and intrathecal injections into CNS. These routes of administration are highly invasive and likely not practical for drugs which need to be given chronically. It is due to the lack of drug-like properties coupled with poor solubility, in vivo instability, and poor penetration across the CNS and cost of manufacture of biologics that has limited their entry into market.

1.2 Intranasal delivery to the CNS

The discovery of obstacle imposed by various protective mechanisms in brain, such as tight junctions of the BBB (Figure 1) and the p-gp efflux pump has increased interest in
developing alternative strategies to overcome these hurdles for delivery of biologics when the need for brain exposure to drugs becomes vital.

In the last few years, the intranasal (I.N) route has emerged as a non-invasive delivery option, which bypasses the BBB and allows direct provision of various biologics-based drugs to the CNS. I.N delivery of therapeutics to the brain was first proposed and patented in 1989 by William H. Frey II of the Alzheimer’s Research Center, both in humans and mammals[1, 2]. In humans, and most other mammals, the nasal cavity is divided into two halves by a septum, with each half comprising three functionally unique regions: vestibular, respiratory, and olfactory (Figure 2). The olfactory epithelium is located just below the cribriform plate of ethmoid bone, which separates the nasal and cranial cavities. Olfactory sensory cells are bipolar neurons, each with a single dendritic process that extends from the cell body to the apical surface of the mucosa, where it terminates in diminutive non-motile cilia. At their basal end, these sensory neurons extend fine non-myelinated axons that form bundles with axons of other olfactory sensory neurons and penetrate the cranial cavity through small holes in the cribriform plate. The exact mechanisms whereby intranasally delivered drugs traverse the olfactory epithelium to reach the brain and cerebrospinal fluid (CSF) are not completely understood; however, it is generally accepted that transport of solutes from the epithelium to brain occurs via three distinct pathways [3] (Figure 3). The first one is the systemic pathway where passage of drug is possible through the respiratory epithelium, after which molecules reach the brain by crossing the BBB. The other two pathways are more concerned with large molecular weight drugs uptake by the brain, such as proteins and nucleic acids; this can occur via: i) intracellular axonal transport by the olfactory sensory neurons, which project into the olfactory bulb of the brain, and ii) paracellular transport through clefts between olfactory epithelial cells in the nasal mucosa.
Trans-neuronal (i.e., intracellular) transport is a slow process that requires endocytosis of the drug by olfactory neurons, followed by transport down the axon. Macromolecules that enter the CNS via the olfactory nerve are known to be distributed in rostral regions of the brain, including the olfactory bulb, anterior olfactory nucleus, forebrain and hippocampus; in contrast, when dissemination of macromolecules occurs via extracellular transport pathways, such as along the trigeminal nerve complex, drugs get distributed in caudal brain regions, including the brainstem, cerebellum and hypothalamus[4].

![Figure 1: Autoradiogram of a mouse following IV administration of radiolabelled histamine showing whole body distribution of the radiolabel, except for the brain and spinal cord](image)

Small, lipophilic drugs are transported most efficiently by the I.N route: a number of such drugs (sumatriptan, zolmitriptan, ergotamine and butorphanol) are available on the market. However, increasing attention is focused on the I.N delivery of biologics (peptides, proteins and genes), as evidence accumulates for their rapid, direct, nose-to-brain transport in both humans and animals. In particular, the uptake of peptides and proteins from the nose is relatively rapid, and is believed to occur via the paracellular route. In one study, uptake of three neuropeptides
(melanocortin 4-10, vasopressin, and insulin) was detected in the CSF of human subjects within 80 minutes of intranasal administration[6]. A number of peptide molecules and growth factors have also been successfully delivered to the brain via the nasal route in animals. For example, IGF-1 given I.N to rats bypasses the BBB and enters the brain, and is distributed along the olfactory and trigeminal pathways; detectable levels are achieved within 30 minutes in rostral brain areas, as well as in caudal and ventral structures (including the midbrain). Nerve growth factor (NGF) has also been successfully administered to rats via this route, and detected in the brain within an hour of delivery[7]. These are just a few examples that illustrate the feasibility of delivering hydrophilic macromolecules to the brain via the nasal route of administration. Once in the brain, distinct anatomical pathways seem to exist for the transport of proteins: thus, Thorne et al., [4] followed the autoradiographic distribution of intranasally administered $^{[125]}$I-insulin-like growth factor-1 (IGF-1) and documented a projection from the olfactory bulb to rostral brain areas, as well as a trigeminal pathway along the trigeminal nerve (from the nasal passages) to the brainstem and spinal cord, carrying the protein to more caudal and ventral areas of the brain.

Figure 2: Anatomical connection’s between the olfactory neurons and the nasal cavity [8]
Drug delivery to CNS via the IN route has been reported[9] in humans, and in animal models of Alzheimer’s disease[10], brain tumors[11], epilepsy[12], pain[13] and sleep disorders. However, it is noteworthy to mention here that we have little understanding of how drug delivery system can be leveraged to enhance nasal residence of the molecules, and how pharmacokinetic profiles of drugs can be altered to afford greater brain exposure – this results in lower exposure in the CNS, despite the needless of passage through BBB and the absence of gastrointestinal and hepatic presystemic elimination.

Figure 3: Schematic diagram showing the proposed pathways by which drugs enter the nasal cavity and pass to brain tissue or into the CSF [4]

1.3 Advantages of intranasal delivery

Traditionally, the nasal mucosa has been utilized as a means for administering drugs in order to access the systemic route for facilitating faster and higher levels of drug absorption. Advantages offered by intranasal drug delivery include:
1) Easy access and needle-free drug application - without the need for trained personnel which facilitates self-medication, and improves patient compliance compared to use of parenteral administration[14].

2) Good penetration of low molecular weight drugs, especially ones that are lipophilic, through the nasal mucosa. For example, the absolute nasal bioavailability of fentanyl is about 80%[15].

3) Rapid absorption and onset of action, due to the nasal epithelium providing a relatively large, highly vascularized absorption surface. The $T_{\text{max}}$ of fentanyl after nasal administration is less than or equal to 7 minutes, which is comparable to use of intravenous (I.V.) injection[15]. Nasal administration of drugs can thus be effectively used for emergency therapies, as an alternative to parenteral administration.

4) Avoidance of the harsh environment of the gastrointestinal tract.

5) Avoidance of hepatic first-pass metabolism, and thus offering the potential for dose reduction compared to oral delivery.

6) Potential for direct delivery of drug to the CNS via the olfactory region, thus bypassing the BBB[16].

### 1.4 Factors influencing nasal drug delivery

The bioavailability of hydrophilic drugs is limited to 10% of amount injected for low molecular weight drugs, and only 1% for high molecular weight drugs like calcitonin and insulin[17] and therefore, peptide and protein therapeutics are underutilized. Here we discuss several challenges associated with the targeting of biologics drugs to the CNS via the I.N route.
A significant limitation to the nasal absorption of polar high molecular weight biologics drugs such as peptides and proteins is low membrane permeability. Drugs can pass the epithelial cell membrane via the transcellular route, by exploiting simple concentration gradients, by receptor-mediated or vesicular transport mechanisms, or by the paracellular route through tight junctions between cells. Polar drugs that are 1000 Da or less in molecular weight generally traverse the membrane via the paracellular route [18] (Figure 4). Tight junctions posses a stringent restriction for large molecule drug transport due to the small size of the channels typically less than 10 Å. Large peptides and proteins can traverse the nasal epithelium by utilizing an endocytotic transport process, but only low amounts can get across in this manner [19]. Attempts have been made in improving the nasal absorption of polar drugs, by co-administration of an absorption-enhancing agents that work by modifying the tight junctions and/or act as an inhibitor of enzymatic degradation [20]. However, the use of such enhancers could be detrimental to the nasal mucosa, so the choice of the absorption-enhancer for a nasally delivered drug that is not easily absorbed must be carefully considered, especially in terms of nasal and systemic toxicity.

Another major factor responsible for the low membrane transport of biological drugs is the mucocilliary clearance mechanism, which rapidly clears the administered drug from nasal cavity. This is a self-clearing mechanism, whereby external agents that bind to the mucus are transported to the nasopharynx and eventually to the gastrointestinal tract [21]. For both liquid and powder formulations that are not bio adhesive (do not adhere to mucus), the half-life for clearance is of the order of 15-30min [22].

Yet another factor that profoundly influences the bioavailability of peptide and protein drugs is enzymatic degradation. The nasal cavity and epithelial cell layer both contain
exopeptidases (such as mono and diaminopeptidases that can cleave peptides at their N and C termini) and endopeptidases, which can attack intra-peptide bonds) [23]. These proteolytic enzymes are believed to be the major barrier against the absorption of peptide drugs, such as calcitonin, insulin and desmopressin [24, 25].

A number of multidrug resistance transporters have also been identified in the human nasal respiratory and olfactory mucosa: these transporters actively export the drug from intracellular to the extracellular compartments, and affect the uptake of biological drugs in the nasal mucosa. P-gp is one efflux transporter that is present on the apical portion of ciliated nasal epithelial cells, and in the submucosal vessels of the human olfactory epithelium [26].

In summary, the I.N route for drug delivery is receiving increased attention because it does not pose restrictions that are similar to those of the BBB; however, the epithelial lining of the nasal cavity has other hurdles including enzymatic degradation, tight junctions, and mucociliary clearance, to the delivery of large molecular weight drugs [27]. Therefore, improving absorption of the drug at the olfactory mucosa is an important consideration when attempting to deliver molecules to the brain via the I.N route.

Figure 4: Paracellular transport of xenobiotics through tight junctions of the olfactory epithelium [28]
1.5 Nanoemulsion as a system for targeting biologic drugs to the brain

The I.N route of delivery is limited by the volume of formulation that can be administered (<400 ul in humans and <100ul in animals); thus, achieving adequate solubility and drug loading in the intended formulation is critical. Simple solutions are rapidly cleared, along with the mucus in the nasal cavity. Although, the I.N route results in a faster rate of absorption, but it is also associated with very short residence times. To improve the CNS delivery of peptides via this route, we encapsulated the therapeutic peptide in naturally occurring oils (from flax seeds or from fish) in a water-based nanoemulsion system. Our formulations also include other excipients like surfactants and naturally occurring lipids.

This particulate system will facilitate the transport of peptide and protein drugs through the nasal mucosa, and protect the drugs from enzymatic activity by increasing the retention time of the drug in the nasal cavity (due to high viscosity); facilitating tight contacts between the nasal mucosa and the drug; and opening the tight junctions between epithelial cells. We plan to use flax seed oil or fish oil that has high levels of omega-3 fatty acids (FAs), which are biodegradable. Omega-3 FAs, also known as essential n-3 polyunsaturated fatty acids (PUFAs), are important to human health, but cannot be produced by the body [29]. Omega-3 FAs play a key role in brain function as well as in normal growth and development, are highly expressed in the brain, and appear to be particularly important for cognitive and behavioral function. Omega-3 FAs are also known to reduce inflammation and minimize risk factors associated with chronic diseases such as heart disease, cancer, and arthritis [30].
1.5 In vitro models for understanding the mechanism of intranasal drug delivery

Increasing numbers of peptide- and protein-based drugs intended for delivery via the I.N route are being developed, and there is a critical need for reliable methods that can be used to study the rate and extent of absorption across the nasal epithelium. While in vivo studies represent the final validation for any nasal drug application or formulation, mechanistic aspects of nasal absorption may be more clearly addressed by use of well-defined and controlled in vitro models, which also limit the number of experimental animals used. Based on the last few years of research, several models have been suggested; each is unique, and together they range from primary cell cultures to use of excised nasal tissue.

Wegner and Kissel [31, 32] developed a primary cell culture model, in which tissues are carefully sampled from particular locations of the nasal cavity, and cells are harvested by various methods. Although recent advances in human nasal primary cell culture techniques appear to be promising, challenges remain with regard to length of culture time, limited access to cells or tissues, and short lifespan of the cultured cells, before this model is usable as a practical tool for investigating drug delivery. To overcome problems with supply of human nasal tissue, the human nasal cell line RPMI 2650, which originated from an anaplastic nasal septum [33], has been employed. This cell line is closely related to the normal human nasal epithelium with regard to its karyotype [34], its cytokeratin polypeptide pattern [35], and the presence of mucous material on the surface of the cells. Excised nasal mucosae from a number of species (rabbit, bovine, canine, human) are frequently used to study nasal transport and metabolism, with rabbit tissue used for the majority of the studies [36]. It has been pointed out that species differences in the activity of nasal enzymes may be an important factor to consider.
Based on findings reported to date, the permeation and metabolic barrier function of excised nasal tissue derived from various animals mimic the in vivo situation to a very high degree. However, the supply of human tissue will continue to be a challenge, and primary cell lines are difficult to handle.

1.6 Current in vivo techniques for evaluation of intranasal delivery

The majority of preclinical work to evaluate intranasal (I.N) delivery has been done in anesthetized mice and rats, with animals positioned in the supine position. Important factors that influence the impact of drug deposition into the nasal cavity after intranasal administration include head position, method of delivery, and volume of delivery. Early studies were also performed with use of radiolabeled drugs or fluorescence-conjugated drugs, to qualitatively determine the uptake of drug in the CNS; however quantitative assessments of concentrations and distributions to different brain areas are needed to gain better insights into the pathways involved in CNS delivery after I.N administration [3]. Drug targeting, or the relative distribution of drug to therapeutic target sites (i.e., brain or specific brain area) compared to exposure to non-target sites (i.e., blood, spleen or other peripheral tissues) is also of great importance in assessing any model of CNS drug delivery. Another consideration is that concentrations observed in the CNS after I.N administration could be due to absorption into the nasal vasculature followed by distribution via the systemic circulation; thus I.V delivery is used as a control to account for blood-mediated distribution into the CNS. Comparing the ratios of brain concentration to blood concentration after I.N and I.V administration should uncover the extent of direct transport to the brain [37-39]. Collectively, these metrics will permit a better comparison of findings from different studies, and a more complete assessment of the effects of formulations on enhancing intranasal delivery to the CNS.
Detailed pharmacokinetic and pharmacodynamics studies in animals are needed to confirm the delivery of the therapeutics to specific areas of the brain [40]. For instance, IGF-1 given intranasally to rats bypasses the BBB, enters the brain, and is distributed along the olfactory and trigeminal pathways; detectable levels are achieved within 30 minutes in rostral brain areas as well as in caudal and ventral structures (including midbrain), and the protein is delivered in quantities sufficient to activate signaling pathways in brain regions known to express the IGF-1 receptor [4]. Intranasally delivered IGF-1 also exerts neuroprotective effects in the rat model of middle cerebral artery occlusion (MCAo) [41] or stroke, where it reduces infarct size and improved neurological function.

1.7 Experimental models of neuro-inflammation

Neuro-inflammation is inflammation of the nervous tissue. It may be initiated in response to a variety of cues, including infection, traumatic brain injury, toxic metabolites, or autoimmunity. In the central nervous system, including the brain and spinal cord, microglia are the resident innate immune cells that are activated in response to these cues. There are a broad spectrum of neurodegenerative diseases, associated with chronic inflammation, such as Alzheimer’s disease, Parkinson’s disease, sclerosis, and all of the tauopathies, and age-related macular degeneration [42]. Although the key molecular and cellular events underlying development of these diseases are clearly divergent, inflammation (which seems to be a common denominator among the diverse list) has recently been implicated as a critical mechanism responsible for the progressive nature of neurodegeneration.

Microglia upon stimulation produces a barrage of factors (IL-1, TNF-α, NO, PGE2, superoxide) that are toxic to neurons. Evidence supports that the unregulated activation of microglia in response to environmental toxins, endogenous proteins, and neuronal death results
in the production of toxic factors that propagate neuronal injury. If the initial stimulus that elicited microglial activation is not resolved (as in the case of a genetic mutation or a prolonged or repeated environmental exposure or immunological insult), a self-sustaining cycle of neuro-inflammation can ensue and such a chronic inflammatory environment is likely to elicit neuronal dysfunction and eventual death of vulnerable neuronal populations. Therefore, timely delivery of anti-inflammatory regimens in patient populations identified to be at risk may afford neuroprotective effects.

One of the strong candidate trigger protein in neuro-inflammation, and thus a potential target for therapeutic manipulation is the potent pro-inflammatory/pro-apoptotic cytokine, tumor necrosis factor-α (TNF-α). It has been demonstrated to play a major role in central nervous system (CNS) neuro-inflammation-mediated cell death in AD, PD and amyotrophic lateral sclerosis (ALS) as well as several other CNS complications. Recently, agents that modulate the levels of circulating peripheral TNF-α protein have been shown to be worthwhile biological therapeutic agents with the use of Enbrel® (Etanercept) and Remicade® (Infliximab), both of which display beneficial properties against rheumatoid arthritis and other peripheral inflammatory diseases. Unfortunately, these agents are largely unable to penetrate the BBB, which severely limits their use in the setting of neuro-inflammation in the CNS.

Cyclosporine, which is a widely used immunosuppressant for transplantation due to its regulation of T-lymphocyte activity, possesses neuroprotective properties because of its ability to block the MPTP, which in turn inhibits neuronal damage [43]. This newly discovered CSA-mediated neuroprotection pathway along with its role in suppressing the immune cells has stimulated research to uncover the potential of CSA in preventing cell death in neurological conditions of Parkinson’s diseases or any other neuro-inflammatory disease. The neuroprotective
benefits of orally administered CSA are evident only with use of a high dose (i.e., >10 mg/kg) of CSA, as well as when drug is delivered chronically; however, such high doses and chronic administration are linked to negative side effects including nephrotoxicity and hepatotoxicity. Thus, the major limiting factor in the therapeutic efficacy of CSA is its ability to cross the blood brain barrier (BBB), pointing to an urgent need for evaluating alternative strategies to improve upon the CNS delivery of CSA [44]. It has been demonstrated that CSA protective effects can be enhanced by mechanical disruption of the brain parenchyma. The other approach taken was to administer endogenous ligands or analogs that can increase permeability of the BBB which can transiently increase the permeability of the BBB.

Our approach is to evaluate the intranasal delivery of cyclosporine peptide and TNF-α specific oligonucleotide to inhibit cytokines stimulation and determine the potential value of anti-inflammatory therapies in a variety of diseases underpinned by inflammation within the nervous system.

1.8 Conclusions

Delivery of biologics offers several advantages for the treatment of brain disorders; however, their delivery to CNS has been facing challenges due to the presence of BBB, blood-CSF barrier, presence of efflux transporters. Intranasal route of administration provides a non-invasive method of by-passing BBB to potentially deliver biologics to CNS. However, the nasal epithelium has some limitation like poor absorption, enzymatic degradation and poor permeation and retention of hydrophilic drugs. In this research study, we investigated on using lipid based delivery system to overcome some of the challenges and to improve upon the CNS exposure.
CHAPTER 2
PREPARATION AND CHARACTERIZATION OF OIL-IN-WATER NANOEMULSION SYSTEMS FOR PEPTIDE AND SMALL INTERFERING RNA (SIRNA) THERAPEUTICS

2.1 INTRODUCTION

As previously mentioned, the primary objective was to develop and characterize nanoemulsion formulations for enhancing the delivery of therapeutic drugs across BBB by exploring the advantage of using intranasal route as method to bypass BBB. We selected two model compounds to study the distribution from nose to brain. We have selected the anti-inflammatory peptide cyclosporine (Figure 5), which is a high molecular weight, lipophilic, cyclic polypeptide consisting of nine amino acids, as one of the model compounds to be investigated. The inherent properties of peptides like enzymatic degradation, high molecular weight, poor membrane transport, poor solubility at high concentration are few factors that could limit its delivery to brain. Previously, cyclosporine peptide has been tested pre-clinically as anti-inflammatory molecule and it showed benefits against stroke and neuro-inflammation in various animal models; however a major challenge has been its poor penetration of the BBB, when dosed via other routes; thus, treatment requires high doses, which leads to side effects. To improve the peptide’s exposure to brain tissue and to resolve some of the limitations for its delivery detailed above we developed nanoemulsion system for intranasal delivery of cyclosporine peptide.
A second anti-inflammatory investigational macromolecule we selected for the research and to evaluate the efficacy against neuro-inflammation was the 21mer oligonucleotide which is specific for TNF-α. TNF-α plays a major role in the progression of neuro-inflammation and is considered one of the major target for treatment therapies. Nucleic acid based drugs such as siRNA have been shown to successfully down regulate the therapeutically important genes recently. While they show highly sequence specific gene silencing behaviors, the in vivo delivery of naked siRNA to brain remains a considerable hurdle. Some of the current pre-clinical trials involve direct ICV administration of siRNA; however this route is highly invasive. Naked delivery of siRNA to brain through intranasal route could be beneficial due to non-invasive delivery and possibility of fast transport. However, due to its high molecular weight, poor enzymatic stability and hydrophilic nature, naked delivery of siRNA is found to be unfavorable for brain delivery. In this regard, engineered nanocarriers that can stably encapsulate/complex protect and selectively deliver siRNAs intracellular are highly promising as next generation gene
delivery vehicles. We therefore, developed and evaluated the cationic based nanoemulsion systems to protect and deliver siRNA intranasally to achieve therapeutic concentrations in brain.

2.2 MATERIALS AND METHODS

High omega-3 fatty acid-containing flaxseed oil was kindly provided by Jedwards International (Quincy, MA). Lipoid E80® was purchased from Lipoid GMBH (Ludwigshafen, Germany). Tween 80®, stearylamine and cyclosporine a peptide was purchased from Sigma Chemicals, Inc. (St. Louis, MO). (N-[1-(2, 3-Dioleooxy) propyl]-N, N, N-trimethylammoniumsalt) (DOTAP) cationic lipid was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). A 21 base pair oligonucleotide against TNF-α gene as obtained from Alnylam Pharmaceuticals (Cambridge, MA) as a gift. SYBR Gold dye and RNase enzyme were obtained from Invitrogen (Carlsbad, CA). All other chemicals were procured from Fisher Scientific (Fair Lawn, NJ) and were used as received.

2.2.1 Preparation of CSA Peptide Nanoemulsion

CSA-containing oil-in-water nanoemulsions were prepared by the sonication method (Figure 6), according to a protocol from our lab [45]. CSA was dissolved in ethanol (50mg/ml): the oil phase consists of flax seed oil with the drug in ethanol and the aqueous phase was prepared by suspending egg phosphatidylcholine (Lipoid E80®). The oil-drug mixture was vortexed for few minutes, and ethanol was completely evaporated with use of liquid nitrogen; the solution was then slowly added to the aqueous phase containing lipoid E80 and tween-80 or stearylamine, the mixture was homogenized, then ultrasonicated in an ice bath to prevent excessive heating of the sample and to protect the peptide from degrading during preparation.
CSA offers limited solubility in water; thus, to compare transport from the nanoemulsion preparation with that from a solution form, we prepared an aqueous suspension formulation that was made with all of the other constituents in nanoemulsion except for the oils. The aqueous suspension was prepared by mixing the CSA in ethanol with deionized distilled water containing egg phosphatidylcholine (Lipoid E80®) and Tween 80 at the same proportions as for the nanoemulsion formulations. Placebo without the cyclosporine was prepared as a control. A screen was performed to select a correct type and percentage of surfactants/excipients (Tween 80, deoxycholic acid, L-histidine, stearylamine) for the nanoemulsion formulation, with the goal of achieving particle size of less than 250nm with low polydispersity.

Figure 6: Outline of the Nanoemulsion formulation process
2.2.2 Determination of nanoemulsion size, surface charge, and morphology

1. Particle Size: Nanoemulsions were characterized for particle size, with use of dynamic light scattering, on Brookhaven Instrument’s 90 Plus ZetaPALS particle size analyzer (Holtsville, NY), at a 90° fixed angle, and at 25°C. Nanoemulsions were diluted with de-ionized water and the z-average of oil droplet hydrodynamic diameter; the polydispersity index (PDI) was recorded; during the measurement, average particle count rate was maintained between 50 and 500 kcps.

2. Surface charge analysis: The ZetaPALS instrument was also used to measure the surface charge (zeta potential) of the nanoemulsions. Measurements were made on diluted nanoemulsions as described above. The refractive index of the nanoemulsion was at 1.33, and the viscosity at 1.0 cps, to mimic the values for pure water.

3. Transmission Electron Microscopy (TEM): The morphology of the oil droplets in the nanoemulsion formulations was visualized with TEM analysis. The nanoemulsion was placed on formvar-coated copper grids (EM Sciences, Hatfield, PA, USA), and negatively-stained at room temperature with 50 μL of 1.5% (w/v) phosphotungstic dye for 10 minutes. Excess liquid was drained off with Whatman filter paper, and the grid containing the dry film of nanoemulsion sample was observed with an EOL 100-X transmission electron microscope (Peabody, MA, USA).
2.2.3 Determination of Cyclosporine (CSA) peptide loading, encapsulation, and stability

Cyclosporine nanoemulsions were analyzed for extent of loading (i.e., amount of peptide incorporated in the internal oil phase of nanoemulsion) with use of high performance liquid chromatography (HPLC). For loading CSA-nanoemulsion was diluted with 100% Methanol and 50μl of the dissolved nanoemulsion was injected on HPLC. The mobile phase A consisting of 1% TFA in water, and mobile phase B as 1% TFA in acetonitrile, was pumped through the Agilent Zorbax 300SB-C18 column (C18, particle size 3.5μm, 4.6mm × 100mm) at a flow rate of 1 mL/min. The gradient was 30% B to 100% B in 8min and peptide elution was monitored at a wavelength of 215nm. For encapsulation efficiency, formulations were first diluted 100 times; of this, 0.5ml was transferred to PVDF Ultra-free centrifugal filter units having 0.1micron pore size (UFC40VV25, Millipore, Bedford, MA) and centrifuged at 5,000g for 15 min at 4°C. The encapsulated drug remains in the donor chamber and the aqueous phase moves through the filter into the sample recovery chamber. The aqueous phase was injected on the HPLC and the concentration of the peptide in the aqueous phase was estimated; encapsulation efficiency was calculated based on mass balance. Nanoemulsions samples were studied for stability with respect to its uniformity (appearance), particle size and surface charge at 3 months after storage at 4°C. Drug loaded nanoemulsions were tested for chemical stability up to 3 months after storage at 4°C.

2.2.4 Preparation of siRNA encapsulated oil-in-water nanoemulsion (SNE)

We investigated the possibility of loading siRNA in nanoemulsion using electrostatic interaction with cationic lipid. In order to prepare siRNA formulation, we used DOTAP (cationic
lipid) to condense and strengthen the attraction of the polyanionic antisense oligonucleotide to the cationic moieties on the oil nano-droplets.

All the formulation optimization work was performed with a control non-target siRNA (NTC) sequence and was finally applied to TNF-α (therapeutic target siRNA). Briefly, in order to incorporate siRNA into nanoemulsion, DOTAP lipid was dissolved in ethanol first and siRNA stock in PBS 1X buffer was added to the DOTAP mixture. The N/P (nitrogen to phosphate) ratio was optimized based on the particle size, zeta, encapsulation of the final nanoemulsion and loading capacity to achieve at least 1mg/ml loading. After condensation of siRNA with DOTAP, oil was added to the mixture to help DOTAP integration in the phospholipid layer. Aqueous phase was then subsequently added to the oil phase after ethanol has been evaporated using nitrogen. Nanoemulsion was homogenized and sonicated for 2min to reduce the particle to low nm range. A screen was performed to select the optimum Nitrogen to phosphate ratio that will provide maximum encapsulation of siRNA.

2.2.5 Characterization of siRNA nanoemulsion

siRNA nanoemulsions (SNE) were characterized for particle size, surface charge and TEM as detailed in above section 2.2.2. SNE were analyzed for encapsulation using gel retardation method and also quantitatively using SYBR green dye. The complexation/encapsulation percentage of siRNA was calculated based on SYBR Gold dye. SYBR gold detects any accessible dsRNA and ssRNA in the formulation which corresponds to free siRNA. Complexed siRNA was detected by the dye after treating the nanoemulsion with triton x-100 which destabilizes the nanoemulsion particles. Encapsulation efficiency was
calculated based on free and total siRNA. The total recovery of siRNA is also calculated to account for any loss of siRNA during the formulation process.

Qualitative assessment of encapsulated siRNA was performed using the gel retardation assay. If siRNA is efficiently bound to DOTAP in the nanoemulsion, migration into the gel will be completely retarded. Further nanoemulsion was treated with a 10% Triton X-100 and run on gel. Triton X-100 destabilizes the particles and hence siRNA released from particles will be able to move through the gel and will be detected as free band in the gel.

To evaluate the protective role of the nanoemulsion against siRNA degradation, SNE were incubated with RNase An enzyme at 37°C for 15min, following which samples were run on gel with 10% triton. Free siRNA was used as a negative control for this study. siRNA standard was run to size the siRNA and visualize any siRNA degradation due to RNase in the gel.

2.3 RESULTS AND DISCUSSION

2.3.1 Preparation of peptide nanoemulsion formulation

Cyclosporine nanoemulsion formulations prepared by sonication method resulted in a uniform milky-white emulsion. Formulations were scaled to encapsulate up to 25-30mg/ml of cyclosporine peptide.

2.3.2 Determination of the oil droplet size, surface charge, and morphology

Particle size and polydispersity index (PDI) were determined for different nanoemulsions prepared by sonication. Table 1 lists the particle size, PDI of different cyclosporine nanoemulsions. The zeta potential for the formulations varied with the increasing concentration of the surfactant. To study the influence of surface charge on the uptake in in vitro and in vivo,
negatively and positively charged nanoemulsions were prepared by modifying the surface using stearylamine (negative charge) or Tween 80 (positive charge). NE-04282012-8(Blue) was selected as the optimal formulation composition, based on the smallest particle size (236nm) and a narrow PDI (0.17).

Table 1: Peptide Nanoemulsion Formulation Characterization

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition</th>
<th>Particle Size (nm) (±SD)</th>
<th>PDI</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE-4282012-1</td>
<td>Lipoid E80/deoxycholic acid 0.88%/flax seed oil</td>
<td>324±15</td>
<td>0.5±0.02</td>
<td>-33±14</td>
</tr>
<tr>
<td>NE-4282012-2</td>
<td>Lipoid E80/deoxycholic acid 1.76%/flax seed oil</td>
<td>310±12</td>
<td>0.40±0.1</td>
<td>-32±20</td>
</tr>
<tr>
<td>NE-4282012-3</td>
<td>Lipoid E80/deoxycholic acid 3.54%/flax seed oil</td>
<td>323±18</td>
<td>0.24±0.05</td>
<td>-31±19</td>
</tr>
<tr>
<td>NE-4282012-4</td>
<td>Lipoid E80/L-histidine 0.88%/flax seed oil</td>
<td>243±12</td>
<td>0.27±0.1</td>
<td>-40±15</td>
</tr>
<tr>
<td>NE-4282012-5</td>
<td>Lipoid E80/L-histidine 1.76%/flax seed oil</td>
<td>291.9±10</td>
<td>0.15±0.2</td>
<td>-51±11</td>
</tr>
<tr>
<td>NE-4282012-6</td>
<td>Lipoid E80/L-histidine 3.539%/flax seed oil</td>
<td>278±09</td>
<td>0.17±0.08</td>
<td>-61±18</td>
</tr>
<tr>
<td>NE-4282012-7</td>
<td>Lipoid E80/Tween 0.88%/flax seed oil</td>
<td>444±30</td>
<td>0.20±0.06</td>
<td>-24±11</td>
</tr>
<tr>
<td>NE-4282012-8</td>
<td>Lipoid E80/Tween 1.76%/flax seed oil</td>
<td>236±20</td>
<td>0.17±0.05</td>
<td>-34±14</td>
</tr>
<tr>
<td>NE-4282012-9</td>
<td>Lipoid E80/Tween 3.539%/flax seed oil</td>
<td>261.9±14</td>
<td>0.19±0.1</td>
<td>-26.5±10</td>
</tr>
</tbody>
</table>
2.3.3 Determination of cyclosporine peptide loading, encapsulation, and stability

An HPLC assay was used to determine the drug concentrations in the nanoemulsion formulations. Loading optimization was performed for CSA peptide to achieve maximum loading for in vivo studies; characterization data shown in Table 2. Based on the encapsulation efficiency, loading of up to 25 mg/ml was achieved for the CSA in positively charged particles with 89% encapsulation efficiency; for negatively charged particles, loading of 30 mg/ml was achieved with an encapsulation efficiency of 87%.

CSA nanoemulsions were found to be physically stable after 3 months at 4°C with major change in particle size or zeta and only a slight increase in polydispersity. Based on HPLC results the drug concentration was similar to initial drug concentration for nanoemulsion and hence we confirmed chemical stability for 3 months at 4°C.

Table 2: Characterization data for the Peptide Formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition</th>
<th>CSA Loading Conc. (mg/ml)</th>
<th>Z-Average (nm)</th>
<th>PDI</th>
<th>Zeta(mV)</th>
<th>% Encapsulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE-01252012</td>
<td>Lipoid E80/ Tween 80/flaxseed oil</td>
<td>25</td>
<td>232±10</td>
<td>0.25±0.06</td>
<td>-33±12</td>
<td>88±10</td>
</tr>
<tr>
<td>NE-01022013</td>
<td>Lipoid E80/Tween 80/Stearylamine/flaxseed oil</td>
<td>30</td>
<td>272±12</td>
<td>0.3±0.09</td>
<td>57±10</td>
<td>88±13</td>
</tr>
<tr>
<td>NE-Blank</td>
<td>Lipoid E80/ Tween 80/flax seed oil</td>
<td>0</td>
<td>260±20</td>
<td>0.38±0.1</td>
<td>-41±8</td>
<td>NA</td>
</tr>
<tr>
<td>CSA-Solution</td>
<td>Lipoid E80/ Tween 80/ethanol</td>
<td>5</td>
<td>366±50</td>
<td>0.49±0.2</td>
<td>-32±19</td>
<td>64.8±19</td>
</tr>
</tbody>
</table>
Figure 7: TEM image of nanoemulsion formulation
NE- 01252012 (Left) and NE- 01022013 (Right). Nanoemulsion size ranges from 100-210nm for positively charged and 150-300nm for negatively charged formulation.

2.3.4 Preparation of siRNA nanoemulsions

The siRNA encapsulated nanoemulsion (SNE) prepared using cationic phospholipid DOTAP by sonication method resulted in a homogeneous milky white emulsion. We optimized the processing conditions and found that a 5min ultrasonication (energy 21%, duty cycle of 25%) resulted in a nanoemulsion with particle size less than 300nm.

2.3.5 Characterization of siRNA-encapsulated nanoemulsion

Nanoemulsions for siRNA were screened for optimum N/P ratio. Optimization of N/P ratio was performed based on the particle size, zeta potential, encapsulation of the final nanoemulsion and loading capacity to achieve at least 1mg/ml concentration (Table 4). Composition of various other excipients is shown here in Table 3.
Table 3: Formulation composition for siRNA nanoemulsion

<table>
<thead>
<tr>
<th>#</th>
<th>Composition</th>
<th>wt./volume %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flaxseed</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Tween 80</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>Lipoid E80</td>
<td>2.32</td>
</tr>
<tr>
<td>5</td>
<td>DOTAP C.L</td>
<td>0.38</td>
</tr>
<tr>
<td>6</td>
<td>siRNA</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 4: Physicochemical characterization data for the siRNA formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>N/P Ratio</th>
<th>Z-average (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>% Encapsulation</th>
<th>% siRNA recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1023SNE#1 NTC</td>
<td>3.72</td>
<td>234.6±12</td>
<td>0.23±0.04</td>
<td>42.1±5</td>
<td>73±10</td>
<td>104±10</td>
</tr>
<tr>
<td>1103SNE#2 TNF</td>
<td>3.72</td>
<td>380.5±15</td>
<td>0.17±0.02</td>
<td>56.5±8</td>
<td>85±12</td>
<td>95±5</td>
</tr>
</tbody>
</table>

The factor that was varied was the amount of DOTAP lipid and siRNA amount was kept constant at 1mg/ml. Results in Table 5 summarizes the characterization data for the study. There was no correlation between particle size and polydispersity with change in DOTAP ratio. However, we found that there was a positive correlation between the increase in the N/P ratio (increasing DOTAP amount) and positive charge on the particles. Formulation with N/P ratio of 3.72 was selected for further \textit{in vitro} and \textit{in vivo} investigations as they showed best encapsulation of siRNA with good particle size distribution. Morphology determination performed using TEM showed the size range from 68 nm to 168 nm for the optimum nanoemulsion (Figure 8). Formulations were also characterized for encapsulation efficiency based on the gel retardation assay. We found siRNA completely associated with particles reflected by the complete
retardation of siRNA in the 2% agarose gels and further it was found that when SNE were treated with a decomplexing agent like triton, siRNA was released from the particles and was able to travel through the gel and was finally visualized as free band using the imager (Figure 9).

Table 5: Characterization data for the SNE Formulations (N/P ratio optimization)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>N/P Ratio</th>
<th>Z-average (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>% Encapsulated (%)</th>
<th>% Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNE#1</td>
<td>7.44</td>
<td>447 ±29</td>
<td>0.24</td>
<td>45±1.8</td>
<td>82.0±5</td>
<td>98.7±5</td>
</tr>
<tr>
<td>SNE#2</td>
<td>3.72</td>
<td>390.2 ±28</td>
<td>0.16</td>
<td>44.2±0.6</td>
<td>70.1±10</td>
<td>102.2±10</td>
</tr>
<tr>
<td>SNE#3</td>
<td>1.86</td>
<td>386.7 ±26</td>
<td>0.52</td>
<td>3.30±0.5</td>
<td>0</td>
<td>20.8±18</td>
</tr>
<tr>
<td>SNE#4</td>
<td>1.86</td>
<td>415.3 ±54</td>
<td>0.28</td>
<td>9.47±0.3</td>
<td>7.9±12</td>
<td>62.3±</td>
</tr>
<tr>
<td>SNE#5</td>
<td>0.93</td>
<td>511 ±84.2</td>
<td>0.362</td>
<td>(-)7.01±0.6</td>
<td>7.4</td>
<td>116.2</td>
</tr>
<tr>
<td>SNE#6</td>
<td>0.465</td>
<td>328.1 ±23.6</td>
<td>0.209</td>
<td>(-)10.2±0.9</td>
<td>0</td>
<td>122.0</td>
</tr>
</tbody>
</table>

Figure 8: TEM image of siRNA nanoemulsion (SNE#2) formulation

Nanoemulsion size for siRNA formulation ranges from 68-168nm.
Figure 9: Electrophoretic retardation analysis of siRNA binding to DOTAP-NE.

*The release of intact siRNA by 10% Triton was shown in NTC siRNA SNE case (Lane 11)*

**Stability of siRNA Formulations:** SNE was found to be stable overnight at 4°C with no phase separation. Further testing of SNE after incubation with RNase enzyme showed that siRNA in nanoemulsion is protected from enzymatic degradation as siRNA bank was found intact during imaging (**Figure 10**).

<table>
<thead>
<tr>
<th>Lane No.</th>
<th>Sample Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 2</td>
<td>std 0.25mg/ml</td>
</tr>
<tr>
<td>Lane 3</td>
<td>std 0.125mg/ml</td>
</tr>
<tr>
<td>Lane 4</td>
<td>std 0.0625mg/ml</td>
</tr>
<tr>
<td>Lane 5</td>
<td>std 0.03125mg/ml</td>
</tr>
<tr>
<td>Lane 9</td>
<td>SNE no triton 0.25mg/ml</td>
</tr>
<tr>
<td>Lane 10</td>
<td>SNE 2% triton 0.2mg/ml</td>
</tr>
<tr>
<td>Lane 11</td>
<td>SNE 10% triton 0.25mg/ml</td>
</tr>
</tbody>
</table>

Figure 10: Electrophoretic retardation analysis of siRNA stability when formulated as SNE

*The incubation of SNE with RNase enzyme showed no major degradation in the siRNA (Lane 2)*
2.4 CONCLUSIONS

Nanoemulsions were reproducibly made by ultrasonication method for both cyclosporine (CSA) peptide and siRNA. In addition we were able to achieve high loading efficiency for CSA peptide in nanoemulsion which indeed would be advantageous for intranasal dosing as high volume is one of the limitation. Moreover, both CSA and siRNA nanoemulsions were found to be stable overtime at 4°C.
CHAPTER 3

IN VITRO EVALUATION OF CELLULAR TRANSPORT, UPTAKE AND CYTOTOXICITY OF THE DELIVERY SYSTEMS USING NASAL EPITHELIAL CELLS

3.1 INTRODUCTION

For peptide and siRNA/oligonucleotide drug delivery, the intranasal route offers an alternative route for non-invasive delivery to target brain. Whereas in vivo studies represent the most crucial test for any nasal drug application or formulation, mechanistic aspects of nasal absorption/permeability may be more clearly approached by well-defined and controlled in vitro studies. We discussed few limiting factors of nasal absorption in the background part of the thesis like physical barrier or poor permeability of large molecular weight drugs, enzymatic barrier of nasal mucosa, efflux transporters etc. Therefore, to understand the barrier properties of the nasal mucosa for the delivery system and to strategize ways to overcome these barriers, we evaluated a number of different in vitro nasal permeability models first. We selected one system based on the trans-epithelial electrical resistance (TEER) values, integrity of monolayer and ease of access to cells for further studying the cyclosporine permeability and to study the effect of formulation excipients in vitro. We evaluated the cytotoxicity profile of the nanocarrier delivery system designed for both molecules – cyclosporine (CSA) peptide and siRNA silencing TNF-α gene before dosing them in vivo for intranasal delivery. For siRNA we evaluated the intracellular uptake in vitro using FACS and confocal microscopy.
3.2 MATERIALS AND METHODS

Human nasal epithelial primary cell line (HNEpC) and growth media were purchased from Promocell® (Germany). Human colon carcinoma (CaCO-2) cells were obtained as a gift from X. Lin, Novartis Institutes for Biomedical Research (Cambridge). Human nasal septum carcinoma cell line (RPMI 2650) was purchased from American Type Culture Collections (ATCC, Rockwell, MD). The media and reagents necessary for culturing CaCO-2 and RPMI-2650 were purchased from Life Technologies (Invitrogen, Carlsbad, CA). Antibodies and reagents for detecting ZO-1 protein expression were also purchased from Invitrogen (Invitrogen, Carlsbad, CA). Transwell® companion plates and inserts were purchased from BD Biosciences (San Jose, CA).

3.2.1 Evaluation of different in vitro cell culture based models to study nasal mucosal drug transport

This section outlines several cell culture models that were investigated for their suitability to study the transport of different molecular weight drug candidates. We assessed the three commercially available nasal cell lines - human nasal epithelial primary cell line (HNEpC), human colon carcinoma (CaCO-2) and human nasal septum carcinoma (RPMI 2650) by evaluating two important parameters: (1) permeability of the paracellular marker compound sodium fluorescein and (2) the integrity of the monolayer (Figure 11). Cell models were further characterized for the expression of tight junction protein zonulae occludens-1 (ZO-1).
Figure 11: Schematic of in vitro model to study the permeability using different cells

1. **Cellular transport and TEER measurements:** For HNEpC transport study, cells were seeded at densities of $10^5$ – $10^6$ cells/cm$^2$ on 4.7cm$^2$ filter inserts made from poly (ethylene-terephthalate) (Falcon, Beckton Dickinson). Sodium fluorescein (SF) permeability and TEER was measured on day 21 after seeding cells on the inserts. CaCO-2 cells were cultured in DMEM/glutamax® medium supplemented with 10% fetal bovine serum, 1% Pen-Strep®, 1% non-essential amino acids, and 1% sodium pyruvate. CaCO-2 cells were seeded at a density of $10^6$ cells/cm$^2$ on the PET inserts. Human nasal septum carcinoma cell line (RPMI 2650-ATCC® CCL30 ™) cultured in a 5% CO$_2$ atmosphere and 95% RH at 37°C. Modified Eagle Medium (MEM) with 20% fetal calf serum, 1% non-essential amino acids, 1% penicillin and 1% sodium pyruvate (Life Technologies) was used as the culture medium. Cells were tested under various conditions for the optimum monolayer formation. The transepithelial electrical resistance (TEER), an indicator of the tight junction formation was measured using an Endohm® EVOM chopstick electrode (WPI, Inc.) every third day after cells were plated to check the integrity of the monolayer overtime. On the day of measuring the permeability using sodium fluorescein, first the cell monolayers were equilibrated with transport media (Hank’s balanced salt solution supplemented with 15mM glucose) for 15 minutes at 37°C followed by final TEER
measurement. Cells were incubated with the stock of sodium fluorescein (paracellular marker) at 5uMolar concentration in the transport media. After the predetermined time points, samples were taken and media was replaced to maintain sink conditions. Sodium fluorescein concentration at various time points was measured using cell plate reader. Sodium fluorescein permeability apparent values (Papp) were calculated based on the following formula:

\[
P_{\text{app}} = \frac{dQ}{dt} \cdot A \cdot C_0
\]

Where, \( \frac{dQ}{dt} \) = amount of product present in basal (A-B) or apical (B-A) compartment in function of time (nmol/s). A = area of filter inserts (cm\(^2\)) C\(_0\) = initial concentration of product applied in apical compartment (nmol/ml).

2. Immunofluorescence assay for ZO-1 tight junction protein expression: To evaluate the expression of TJ proteins, immunofluorescence protocol (modified Invitrogen protocol for IF staining tight-junctions protocol) was performed. Cells were seeded on glass coverslips placed in 6-well plastic plates (Costar, Inc.) and grown for 3-5 days; they were washed with cold PBS, pre-extracted for 2min on ice, washed again with cold PBS, and fixed in cold 3\% paraformaldehyde in PBS for 10min on ice. Cells were permeabilized with 0.05\% triton in PBS for 2 min; non-specific staining was blocked by immersion for 1hour in a solution of bovine serum albumin (BSA). Cells were incubated overnight at 4°C with primary antibody (anti-ZO-1- anti rabbit from Invitrogen, Inc.), washed in PBS, and then incubated with secondary antibody (anti-rabbit - Alexa flour 488, Invitrogen) for 1 hour on ice. Fluorescence was measured after fixing the cells with Prolong anti-fade mounting media containing the nuclear stain DAPI (Invitrogen).
3.2.2 Investigation of effect of formulations on permeability and Intracellular uptake of cyclosporine in RPMI 2650 cell monolayer

We selected RPMI2650 cell model for evaluating and comparing the nasal permeability of molecules in vitro. Firstly, we conducted a study using RHO 123 as a surrogate molecule due to ease of measuring (the fluorescence of) the drug at different time points, and also due to similarity in lipophilicity to the CSA peptide. Rho 123 transport at various time points was measured on the basal side of the monolayer using the absorption at 470nm wavelength. Papp values were calculated for the both treatments. Further, to study the impact of formulations on the permeability of CSA peptide we compared the solution form of CSA to nanoemulsion negatively-charged and positively-charged nanoemulsion formulations. The RPMI-2650 cells were cultured on inserts at a density of 300k cells/cm² which are kept in flat bottomed 12-well plates. Media was changed every third day until 7 days and after 7 days cells were grown on the air-liquid interface for another 7 days. TEER was measured after 14 days and cells were fed with either 20uMolar of CSA in nanoemulsion form (positively or negatively charged) or with CSA solution (in 0.5% DMSO HBSS 1X buffer) at similar concentration (n=3). Samples were collected after 3 hour time point and CSA concentration was analyzed using LC-MS system. To evaluate and compare the intracellular uptake of CSA from nanoemulsion formulations versus the solution from of CSA peptide, RPMI2650 cells were cultured on inserts at a density of 300k cells/cm² which are kept in flat bottomed 12-well plates. Media was changed every third day until 7 days and after 7 days cells were grown on the air-liquid interface for another 7 days. Trans-epithelial electrical resistance (TEER) was measured after 14days and cells were fed with either 20 µMolar of CSA in Nanoemulsion form (Positive and negative charged) or with CSA solution (in 0.5% DMSO HBSS 1X buffer) at similar concentration (n=3). After 3 hours and 24
hours of incubation, cells were washed 3 times with cold 1x PBS and cell lysis buffer was added to the wells and cell lysate was collected from respective wells. CSA was extracted using solvent extraction method. Briefly, 500 µl of acetonitrile was added and after mixing cell debris was collected, centrifuged and 800 µl of supernatant was aliquoted out in new tube and dried by solvent evaporation. Dry sample was re-dispersed with 200 µl of 50/50 of LC-MS media buffer B (5 mM ammonium formate in methanol) and acetonitrile. Samples were run on LTQ LC-MS system with a standard curve prepared from cyclosporine. The amount of total protein was determined using a BCA protein assay kit (Thermo Scientific) and analyzed using a fluorescent plate reader.

3.2.3 Evaluation of intracellular uptake of siRNA nanoemulsion using confocal microscopy and flow cytometry studies

Site-specific delivery of small interfering RNAs (siRNAs) and intracellular uptake of siRNA still remains to be a major hurdle that needs to be addressed before delivery of siRNA can become clinically viable therapeutic option. We therefore wanted to understand the uptake of TNF-α siRNA formulation (SNE) in vitro in J774.A.1 macrophages before we could evaluate them for intranasal delivery in vivo or even so before we could evaluate their anti-inflammatory efficacy in LPS stimulated macrophages. To evaluate the uptake and cellular internalization of the naked siRNA (as control), we first encapsulated cy3-siRNA label siRNA (Invitrogen) into the nanoemulsions using similar protocol as described above for TNF-α siRNA formulation. Naked Cy3-labeled siRNA, Lipofectamine Cy3-siRNA, SNE (siRNA nanoemulsions) were incubated with 200nMolar J774A.1 macrophages in 6 well plate in the presence of DMEM supplemented with 10% FBS. Quantitative uptake analysis of SNE was carried out with Cy3-siRNA Lipofectamine complex and Cy3-siRNA encapsulated in Nanoemulsion by flow
cystometry using BD Biosciences FACS caliber (San Jose, CA) after 15min and 2.5 hour time point for incubation with cells. The FL-2 channel (585/42 emission) was used to detect the cells containing Cy3-dye label particles. A total of 10,000 events were counted within a gated region. The results obtained were analyzed using Cell-Quest Pro-software. The data is presented as % uptake compared to control untreated cells. Additionally, at pre-determined time points 1 hour and 2 hour post administration of the nanoemulsion samples, glass cover-slips placed in the 6-well microplate were removed and rinsed with sterile PBS and inverted on a clean slide for qualitative analysis of uptake and cellular internalization using fluorescence microscopy. Lysotracker green (Invitrogen) was used for labeling lysosomes in the cells and Hoechst (Blue) was used to label the nuclei of cells. Bright field and fluorescence images were acquired with a BX51-TRF Olympus (Center Valley, PA) inverted microscope at 40× original magnification.

3.2.4 Evaluation of in vitro tolerability of cyclosporine and siRNA nanoemulsion formulations

For CSA peptide tolerability studies, RPMI 2650 cells were seeded at a density of 5000 cells/well in 96 wells; after 72 hours, cells were rinsed and incubated with different % v/v treatment solutions: CSA-nanoemulsion, CSA solution, and blank nanoemulsions, diluted with the cell media. Treatment with cell media was used as a negative control and treatment with poly(ethylenemine) (PEI, M.wt. 10kDa), a cationic cytotoxic polymer, was used as a positive control. Following 24 and 72 hours of treatment time, the cells were rinsed with media and 50μl of MTT reagent (Vybrant® MTT Cell Proliferation Assay Kit, Life Technologies) was added. During the incubation period of 1 hour at 37°C, MTT dye was converted to formazan by the live cells. DMSO 200μl was added to the plates and after shaking for few minutes absorbance was
measured at 490nm. The absorbance value is directly proportional to the remaining living cells. Similarly, cytotoxicity analysis of the delivery system for TNF-α siRNA was also performed using MTT Reagent. Various concentration of NTC-siRNA containing nanoemulsion ranging from 2nM to 100nM of two different formulations was tested with 10,000 J774A.1 adherent murine macrophages in 96-well micro plates. Cell cytotoxicity study was performed at an early time point – 2 hour and 19 hours. After washing with sterile PBS, the wells were treated with MTS reagent for 2 hour and the absorbance of the chromogenic formazan product in viable cells was measured with a 490 nm BioTek Synergy® HT microplate reader. The percent cell viability was calculated from the absorbance values relative to those of untreated cells. The samples were tested with n = 8 replicates.

3.3. RESULTS

3.3.1 Evaluation of different in vitro cell culture based models to study nasal mucosal drug transport

HNEpC cells monolayer were ready for permeability studies in 3 weeks. Even though the tight junctions expressed (Figure 13) in the nasal mucosa layer were present in the HNEpC cells, sodium fluorescein (SF) permeability was found to be one order higher (2*10^5 cm/sec) than normally recorded for a paracellular marker (Figure 12). The high permeability of SF could be due to the low TEER value recorded for these cell monolayers. Furthermore, this model had other major limitations, including slow growth and short lifespan of the cells, which in turn delayed access to lot of cells needed for the transport studies.
Figure 12: Sodium fluorescein permeability and monolayer integrity as measured by TEER in HNEpC cell model

Figure 13: Immunofluorescence for anti-ZO-1: Presence of tight junctions (green) on the HNEpC.

The panel on the left top is the bright field image, and to the right is the fluorescence overlay (40x).

The CaCO-2 *in vitro* cell model has been used for years to predict the absorption of orally administered drugs in the intestinal epithelium. We wanted to investigate if this model could be
used to study the permeability of high molecular weight peptides for nasal transport. CaCO-2 cells formed uniform monolayers when cultured on PET inserts in 12well transwell plates (1.0micron pore size). Sodium fluorescein (SF) permeability and TEER was measured on day 21 after seeding cells on the inserts. SF permeability values ($5\times10^{-6}$ cm/sec) (Figure 14) were in agreement with reference values in literature. Furthermore, TEER values, which signified the integrity of the membrane, were also high (800ohm*cm$^2$). Expression of the tight junction protein ZO-1 was also confirmed as shown (Figure 15). This model can act as a surrogate model for the understanding of the compounds absorption, due to the right characteristics offered – easy setup, high TEER, low permeability of the paracellular marker and expression of tight junction protein ZO-1. However, a major limitation is the lack of similarity to nasal mucosa, as the cells are derived from the colon.

Figure 14: Sodium fluorescein permeability and monolayer integrity as measured by TEER in CaCO-2 cell model
Figure 15: Immunofluorescence for anti-ZO-1: Presence of tight junctions (green) on the CaCO-2 cells.

The panel on the top left is the bright field image and to the right is the fluorescence overlay (40x).

The third cell model with RPMI2650 cells formed a uniform confluent monolayer when cells were grown under an air-liquid interface. Highest TEER values (200ohm*cm²) were observed when cells were seeded at a density of $4 \times 10^5$/cm² onto a PET insert of 0.4cm² surface area with 0.4 μm pore size, which demonstrates the barrier-like properties of the model. RPMI 2650 cells also showed the presence of the tight junction protein ZO-1 (Figure 17). Furthermore, the apparent permeability coefficients of the paracellular marker sodium fluorescein was found to be $8.07 \pm 0.01 \times 10^{-6}$ cm/s (Figure 16), which is indication of uniform monolayer.
Figure 16: Sodium fluorescein permeability and monolayer integrity as measured by TEER in CaCO-2 cell model.

Figure 17: Immunofluorescence for anti-ZO-1: Presence of tight junctions (Green) on the RPMI2650 cells.

*The panel on the left top is the bright field image and to the right is the fluorescence overlay (40x).*
The data on cell characterization indicated that RPMI 2650 cells that grow at an air-liquid interface forms polarized monolayers, with cells interconnected by tight junction proteins. This cell line model for human nasal epithelium is a useful tool for in vitro screening of nasal drug candidates. To validate the model further we thus conducted a study to compare the permeability of rhodamine 123 (Rho 123) and CSA peptide in RMPI 2650 cell model.

### 3.3.2 Investigation of effect of formulations on permeability and intracellular uptake of cyclosporine in RPMI 2650 cell monolayer

Rho 123 showed higher permeability when delivered as a nanoemulsion (NE-Tween) compared to when it was delivered as an aqueous solution (Figure 18). This suggested that nanoemulsion formulation has an impact on transport mechanism of the molecule. Further, we investigated the permeability of CSA peptide in the RPMI 2650 cell monolayer model to check if similar results will be obtained.

![Figure 18: Apparent permeability value of the RHO 123 solutions versus nanoemulsion](image)

**Graph showing a 2 fold increases in Papp of the nanomulsions when compared to solutions form.**
The results showed increase in permeability of CSA with NE-CSA particles compared to Solutions (T-CSA) or NE-Tween formulation (Figure 19). Positively charged particles reduced the efflux ratio from 2.88 to 0.01, which suggests CSA peptide efflux due to the P-glycoprotein (Pgp) and other efflux transporter is reduced when CSA is encapsulated in lipid-based nanocarriers.

![Permeability at 3 hours](image)

**Figure 19:** Permeability of cyclosporine in RPMI2650 cells from AB and BA direction.

*Cells were delivered cyclosporine in positive (NE-SA) and negative charged (NE-Tween 80) nanoemulsion and control solution of cyclosporine with 0.5% DMSO for 3 time point. Efflux ratio with the Nanoemulsion was found to be lower compared to solution formulation.*

**Table 6:** Comparison of Efflux Ratio for Nanoemulsion formulations in RPMI2650 permeability model

<table>
<thead>
<tr>
<th></th>
<th>AB ng/ml</th>
<th>BA ng/ml</th>
<th>Efflux ratio BA/AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-CSA</td>
<td>100.54</td>
<td>289.700</td>
<td>2.88</td>
</tr>
<tr>
<td>NE-SA</td>
<td>582</td>
<td>8.570</td>
<td>0.01</td>
</tr>
<tr>
<td>NE-T</td>
<td>115</td>
<td>BLOD*</td>
<td>0.00*</td>
</tr>
</tbody>
</table>
Intracellular studies performed with CSA peptide showed similar intracellular uptake for this peptide at early time point. For solutions S-CSA (solution) and T-CSA (in 0.5% DMSO and ethanol) uptake seems to be saturated, on the other hand for the Nanoemulsion uptake kept increasing at later time points. This can be explained due to the efflux transport of CSA peptide. There was no clear difference for intracellular uptake for NE-SA (+ve charged) and NE-Tween formulation (-ve charged).

Figure 20: Intracellular uptake of cyclosporine in RPMI-2650 cells.

Cells were delivered cyclosporine in positive (NE-SA), negative charged (NE-Tween 80) nanoemulsion, control solution of cyclosporine with no oil and with 0.5% DMSO for 3 and 24 hours’ time point.

3.3.3 Evaluation of intracellular uptake of siRNA nanoemulsion using confocal microscopy and flow cytometry studies

To quantitative and qualitative analyses of Cy3-Label siRNA were evaluated using flow cytometry and fluorescence microscopy, respectively. Figure 21 shows the flow cytometry results of nanoemulsions uptake in J774A.1 murine macrophages after 15min and 2.5hour time points of incubation. The CY3-siRNA delivered with Lipofectamine did not show a significant
uptake compared SNE (siRNA nanoemulsion). The fluorescence microscopy images further confirmed that nanoemulsion internalization results of flow cytometry. A significant higher intracellular accumulation of the Cy3-label siRNA DOTAP nanoemulsion as compared to Cy3-siRNA control delivered with Lipofectamine (a commercially available transfection agent).

Figure 21: Cellular uptake of the CY3-siRNA containing nanoemulsion particles in J774A.1 macrophages. 

Figure shows the flow cytometry results of nanoparticle uptake in cells after 15min and 2.5hour of incubation. The FL-2 channel (585/42 emission) was used to detect the cells containing CY3-label siRNA containing particles (A). Flow cytometry data presented as % fluorescence intensity/uptake compared to control untreated cells (B). Fluorescence microscopy images showing the overlay red, blue, green images for siRNA in Lipofectamine compared to SNE at 200nM concentrations. The images were taken at 40× original magnification(C).
3.3.4 Evaluation of cyclosporine and siRNA nanoemulsion tolerability in-vitro

Nanoemulsion formulations of CSA showed enhanced tolerability up to a concentration of 20uMolar as compared to the solution form of the peptide which showed viability of only 50% under similar conditions, signifying that the nanoemulsion uptake being endocytosis might not cause cell toxicity compared to CSA peptide which can interact with the cell membrane directly and cause cytotoxicity.

![Tolerability Study in RPMI 2650 Cells](image1)

![Tolerability study at 48hours](image2)

Figure 22: RPMI 2650 Cell viability results for CSA nanoemulsion formulations when compared to solution of CSA (at 24 and 48hrs)
siRNA encapsulated nanoemulsion (SNE) formulation with or without PEG showed almost 100% cell viability both at early time point and later time point inferring no potential cytotoxicity concerns from the novel cationic delivery system in vitro.

Figure 23: Cytotoxicity of siRNA nanoemulsion (SNE) at 2 hour and 1 hour time point in J774A.1 macrophages.

The cell viability of the untreated cells was considered to be 100%, and the values obtained in the rest of the treatment groups were normalized to control values and presented in percentage form. The values reported are mean ± SD (n = 8).
3.4 CONCLUSIONS

Our *in vitro* characterization findings suggest that it is possible to form a permeation model of nasal mucosa with RPMI2650 cells, especially at air-liquid interface, which permits a confluent growth. We used RPMI2650 cell model to study the permeability of cyclosporine and to study the impact of formulations on cell transport. Efflux ratio of cyclosporine was considerably reduced when encapsulated in NE-CSA versus when delivered in solutions form suggesting an advantage of nanoemulsion in bypassing the efflux transporter and improving permeability. Furthermore, increase in intracellular uptake was found for NE-CSA formulations especially at later time which further confirms that cyclosporine transport through efflux transporter is reduced by the use of oil-in-water nanoemulsion particulate system. SNE (siRNA encapsulated nanoemulsion) also showed a significant impact on the intracellular uptake as evident both qualitatively using confocal or quantitatively using flow cytometry results. Furthermore, cyclosporine and siRNA nanocarriers system were well tolerated in the murine macrophage cells. Based on invitro exploration, we believe these delivery systems show great promise in vivo for intranasal delivery due to effect on uptake and transport of the peptide and siRNA.
CHAPTER 4

IN VITRO EVALUATIONS OF ANTI-INFLAMMATORY EFFECTS OF CYCLOSPORINE (CSA) AND SIRNA NANOEMULSION IN LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGE MODEL

4.1 INTRODUCTION

In order to evaluate and compare preliminary anti-inflammatory effect of cyclosporine and TNF-α silencing small interfering RNA (siRNA) duplex formulations we used J774A.1 adherent murine macrophages stimulated with lipopolysaccharide (LPS) as an experimental model to test the protective role of these therapeutic agents. These cells were used as surrogate cell model due to limited supply/source of primary microglial. Bacterial LPS has been extensively used in models studying inflammation as it mimics many inflammatory effects of cytokines, such as up regulation of cytokines like TNF-α, IL-1β or IL-6. LPS is the most abundant component within the cell wall of Gram-negative bacteria. It can stimulate the release of inflammatory cytokines in various cell types, leading to an acute inflammatory response towards pathogens [46]. LPS after binding to toll-like receptor 4 (TLR4) leads to activation of NF-κB expression which regulates the transcription of genes related to innate immunity and inflammation responses (Figure 24).
Figure 24: Simplified schematic representation of the link between LPS-induced activation, inflammatory mediators and dopaminergic neurodegeneration [47]

4.2 MATERIALS AND METHODS

The Taqman probe/primer for target gene IL-1β, IL-6, iNOS, TNF-α were ordered from Invitrogen with a FAM label and β-actin endogenous gene probe/primer set was ordered with VIC probe label for distinction from the target gene detection. RNA isolation kit was ordered from RNA isolation kit (Roche, Indianapolis, I.N.).

4.2.1 Evaluation and comparison of anti-inflammatory efficacy of cyclosporine nanoemulsion in J774A.1 adherent murine macrophage cell line

To quantitatively assess the potential anti-inflammatory therapeutic effect of the CSA loaded nanoemulsion, the levels of pro-inflammatory cytokines tumor necrosis factor α (TNF-α), Interleukin (IL-1β), Interleukin (IL-6) and the inducible nitric oxide synthase (iNOS) was measured at the mRNA levels by RT-PCR and protein levels were measured only for TNF-α.
gene in lipopolysaccharide (LPS; Sigma Aldrich) stimulated J774A.1 macrophages cells. Cells were counted and plated on day one in a 6-well micro plate at 50k cells density and after day 4 cells were first treated with cyclosporine solution or with Nanoemulsion formulation for 4 hours (n=3) at a concentration of 1ug/ml. After 4 hours of pre-treatment, cells were stimulated with 100ng/ml concentration of LPS. Following 6 hours of stimulation, supernatant was collected and cells were washed and collected by centrifuge at 5,000g for 10min. Cell pellet was used for RNA extraction using the manufacture protocol (Roche, Indianapolis, I.N.). RNA quantitation and integrity test was performed before use in RT step. Gene quantitation assays using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays are performed in a two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from RNA.
2. In the PCR step, PCR products are quantitatively synthesized from cDNA samples using the TaqMan Gene Expression Master Mix.
Figure 25: The figure illustrates two-step PCR scheme followed for the quantitation of target gene expression

Approximately, 400ng of RNA was used for the cDNA synthesis in the first step of RT (performed using Taqman Reverse transcription reagents). The second step qPCR was performed using the taqman probes for the specific cytokines using 5ul of the total cDNA prepared in first RT step with total number of 40 cycles run for each gene. Samples were run in duplicates for β-actin, IL-β, IL-6, TNF-α and iNOS gene. Standard curve was also prepared using cDNA from the LPS treated cells. Samples were analyzed by relative quantitation method by calculating ddcot values for each treatment. TNF-α levels were measured at protein level by ELISA kit for TNF-α (R &D Systems).
4.2.2 Evaluation of gene silencing of siRNA nanoemulsion in LPS stimulated macrophages as an inflammation model

Among various pro-inflammatory cytokines involved in the pathogenesis of neuro-inflammation, tumor necrosis factor (TNF-α) plays a pivotal role in the release of other cytokines and induction of chronic neuro-inflammation. Small interfering RNA mediated knockdown of proinflammatory cytokines at the messenger RNA (mRNA) level offers an alternative therapeutic strategy to overcome neuro-inflammation. Even though sienna has a therapeutic potential, they have a challenge to be delivered intracellularly due to their high molecular weight, enzymatic instability, in physiological fluids. Here we designed a Nanoemulsion system containing siRNA where siRNA has been precomplexed with DOTAP lipid. We evaluated the anti-inflammatory efficacy of TNF-α silencing siRNA in J774A macrophages before we could evaluate the *in vivo* distribution and knockdown in an LPS induced model of neuro-inflammation in rats. Cells were plated in 6 well plates at 200k density on day 1 and on day 2 cells were treated with siRNA nanoemulsion at various concentrations and compared to treatment with siRNA delivered with Lipofectamine cationic lipid transfection agent. We used a non-targeted siRNA incorporated in nanoemulsion as a negative control. RNA extraction was performed using RNA protocol followed by integrity check and quantitation of RNA. Two steps RT-qPCR was performed using Taqman probe and primer set specific for TNF-α. Samples were analyzed by relative quantitation method by calculating ddct values for each treatment.

4.2.3 Statistical Analysis

The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test.
4.3 RESULTS

4.3.1 Evaluation and comparison of anti-inflammatory effects of cyclosporine nanoemulsion in surrogate J774 macrophages cells

The potential anti-inflammatory effect of NE-CSA in J774A.1 macrophages was evaluated by measuring the levels of TNF-α, IL-1β, IL-6 and iNOS proinflammatory cytokines, expressed in macrophages following stimulation with LPS toxin. First we determined the cytokines which showed stimulation with LPS. Previously, we have seen that stimulation of cells with LPS for 4 to 6 hour time point results in high expression of these cytokines. We selected 4 hour as a pre-treatment time point for the CSA nanoemulsion (CSA-NE) and CSA solution (CSA-S). RNA extracted from the cells after treatment was found to be of good integrity and quality. The slope and regression values for all the individual cytokines prepared using the LPS treated cDNA is shown in Table 7 below. All four cytokines standard curves were found to be linear with a good slope value. When comparing the solution of CSA (1ug/ml) to NE (1ug/ml) formulation, there was a significant inhibition found on the levels of all the four cytokines (Figure 26 to Figure 29). Solution formulation at this concentration tested showed less of an effect on the cytokines possibly due to the high efflux of CSA solution form which was evident in the permeability studies performed with nasal squamous epithelial cells.

Table 7: Regression curve analysis data for different cytokines prepared for quantitation of gene knockdown in RT-qPCR assay

<table>
<thead>
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<th>Cytokine Type</th>
<th>Slope</th>
<th>Intercept</th>
<th>R2</th>
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<td>0.997107</td>
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<td>m-TNF</td>
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<td>27.863609</td>
<td>0.996725</td>
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<td>m-IL-6</td>
<td>-2.863811</td>
<td>31.91925</td>
<td>0.994999</td>
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<tr>
<td>iNOS</td>
<td>-3.232734</td>
<td>32.94072</td>
<td>0.996673</td>
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</table>
Figure 26: Inflammatory marker iNOS specific mRNA results showing iNOS transcript expression in the J774A.1 macrophage cell line, comparing the treatment with cyclosporine solution and CSA-loaded nanoemulsion.

Data was plotted by considering the iNOS expression as 100% in untreated cells that were not given any treatment. The values reported are mean ± SD (n = 3). The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test.
Figure 27: TNF-α specific mRNA (left) and ELISA (right) results showing mTNF-α cytokine expression in the J774A.1 macrophage cell line comparing the pre-treatment with CSA-solution (CSA-S) and CSA-loaded nanoemulsion (CSA-NE).

Data was plotted by considering the TNF-α expression as 100% in untreated cells that were not given any treatment. The values reported are mean ± SD (n = 3). The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test with a 99% confidence interval (p < 0.001). For TNF-α mRNA levels statistical significance was determined using one way ANOVA and Sidak’s multiple comparisons test was performed for comparing LPS group to LPS+CSA-NE group.
Figure 28: Inflammatory marker IL-1β specific mRNA results showing IL-1β transcript expression in the J774A.1 macrophage cell line comparing the pre-treatment with cyclosporine solution (CSA-S) and CSA-loaded nanoemulsion (CSA-NE).

Data was plotted by considering the IL-1β expression as 100% in untreated cells that were not given any treatment. The values reported are mean ± SD (n = 3). The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test.

Figure 29: Inflammatory marker IL-6 specific mRNA results showing IL-6 transcript expression in the J774A.1 macrophage cell line comparing the treatment with cyclosporine solution and CSA-loaded nanoemulsion.
Data was plotted by considering the IL-6 expression as 100% in untreated cells that were not given any treatment. The values reported are mean ± SD (n = 3). The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test.

4.3.2 Evaluation of gene silencing of siRNA nanoemulsion in LPS-stimulated macrophages as an inflammation model

Macrophages cells upon stimulation with LPS resulted in substantial increase in levels of TNF-α cytokine. Using anti-TNF-α siRNA, gene knockdown was determined by measuring the level of TNF-α gene expression using RT-qPCR method. Standard curve and slope results are shown in the Table 8.

Table 8: Regression curve analysis data for mouse TNF-alpha cytokine prepared for quantitation of gene knockdown in RT-qPCR assay

<table>
<thead>
<tr>
<th>Detector Name</th>
<th>Slope</th>
<th>Intercept</th>
<th>R2</th>
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<td>mb-actin</td>
<td>-3.46136</td>
<td>27.52513</td>
<td>0.999028</td>
</tr>
<tr>
<td>m-TNF</td>
<td>-3.36661</td>
<td>26.88159</td>
<td>0.999662</td>
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</tbody>
</table>

Oil-in-water nanoemulsion containing TNF-α silencing siRNA showed dose dependent silencing efficiency which was found to be higher compared to siRNA delivered with Lipofectamine transfection agent. It is noteworthy to mention that Lipofectamine induced similar silencing at an earlier time point of 12 hours and duration of effect at 24hour time point was unnoticeable however, Nanoemulsion showed a longer duration of TNF-α silencing up to 24hours of siRNA treatment (Figure 30-31).
Figure 30: TNF-α gene knockdown result in LPS stimulated macrophages showing inhibition of TNF-α mRNA expression in the J774A.1 macrophage cell line

Graph comparing the treatment with TNF-α silencing siRNA dosed with Lipofectamine transfection agent, with Nanoemulsion containing TNF-α siRNA at 12 hour of pretreatment and LPS stimulation for 6 hours. Data was plotted by considering the TNF-α expression as 100% in LPS stimulated cells. The values reported are mean ± SD (n = 3).

Figure 31: TNF-α gene knockdown result in LPS stimulated macrophages showing inhibition of TNF-α mRNA expression in the J774A.1 macrophage cell line

Graph comparing the treatment with TNF-α silencing siRNA dosed with Lipofectamine transfection agent, with nanoemulsion containing TNF-α siRNA at 24 hour of pretreatment and LPS stimulation for 6 hours. Data was plotted by considering the TNF-α expression as 100% in LPS stimulated cells. The values reported are mean ± SD (n = 3).
4.4 CONCLUSIONS

This work demonstrates the potential therapeutic approach of reducing inflammatory cytokines which are involved in the pathogenesis of neuro-inflammation using nanoemulsion system loaded with cyclosporine peptide and TNF-α silencing siRNA. NE-CSA were found to show better anti-inflammatory efficacy compared to the solutions form of cyclosporine when tested in macrophages stimulated with LPS which can be explained by the fact that nanoemulsion system are being able to bypass efflux transporter and can lead to high intracellular accumulation compared to solution form of the peptide. Similarly, TNF-α siRNA containing nanoemulsion showed better silencing of TNF-α gene in macrophages when compared to siRNA transfected with Lipofectamine due to the higher intracellular uptake and trafficking possible and shown in the earlier experiments with siRNA nanoemulsion. Due to their positive effect on the various cytokines we further evaluated these formulations in vivo in an LPS stimulated neuro-inflammation model either in combination or alone.
CHAPTER 5

QUALITATIVE EVALUATIONS OF INTRANASAL NANOEMULSION TRANSPORT TO THE BRAIN USING MAGNETIC RESONANCE IMAGING (MRI) IN RATS

5.1 INTRODUCTION

Intranasal delivery offers an alternative non-invasive strategy to target brain diseases. The olfactory region of the nasal cavity has anatomic and physiologic attributes that provide both extracellular and intracellular pathways into the CNS [20]. The aim of the present study was to investigate the potential delivery of nanoemulsion formulation directly into the CNS following intranasal (I.N.) administration and to understand the distribution into different regions of brain. Further, we want to elucidate if different pathways are involved and assess the potential advantages of using nanocarrier based system rather than using aqueous formulations for intranasal delivery to brain. Nanocarriers offer several advantages for various biologics like higher residence time, high loading, and biocompatibility. These systems are known to provide protection from enzymatic degradation and also can bypass efflux due to efflux transporters. A direct extracellular pathway between the nasal passages and the brain was first conclusively demonstrated for horseradish peroxidase (HRP), a 40 kDa protein tracer [48]. Electron microscopy showed that intranasal administered HRP migrates through open intercellular clefts of the olfactory epithelium to the olfactory bulbs of mice, rats and squirrel monkeys within minutes after application. Conversely, the intracellular pathway from the nasal passages to the brain (i.e. anterograde axoplasmic transport within olfactory sensory neurons) has been
demonstrated most convincingly for the lectin conjugate wheat germ agglutinin-HRP (WGA-HRP). Following I.N. administration, WGA-HRP, unlike HRP, undergoes adsorptive endocytosis into olfactory sensory neurons and subsequent transcytosis to olfactory bulb glomeruli [49], a process that requires 6 h in mice. Electron microscopy demonstrated that WGA-HRP does not undergo extracellular transport into the olfactory bulb from the nasal passages, in contrast to HRP [48].

The purpose of this comparative study was to compare gadolinium encapsulated-nanoemulsion and N-methylglucamine salt of the gadolinium complex of diethylenetriamine pentaacetic acid (Magnevist®) aqueous solution uptake into brain using magnetic resonance imaging (MRI) which is being considered the gold standard in the diagnosis of brain disorders and brain imaging.

5.2 MATERIALS AND METHODS

Gadolinium (III) chloride hexahydrate (Gd3+), diethylenetriamine pentacetic acid (DTPA), Arsenazo dye were purchased from Sigma Aldrich (St. Louis, MO).

5.2.1 Synthesis and purification of phosphatidylethanolamine (PE) conjugated to diethylenetriamine pentacetic acid (DTPA), in order to chelate Gd3+ ions for enhancing MRI contrast

To study the distribution of nanoemulsion in brain after intranasal dosing, we first prepared contrast agent containing formulations. Contrast agent for MRI have unpaired electrons that interact with surrounding water molecules to decrease their proton relaxation time(T1). MRI can measure T1 by creating a magnetic field that reverses the sample’s magnetization, then
recording the time required for the spin directions to realign in their equilibrium positions, a
decrease in the T1 relaxation time of the target tissue allows MRI instrument to better distinguish
contrast from surrounding aqueous environment.

To prepare the nanoemulsion with gadolinium ions for contrast enhancement in MRI, we
synthesized and purified DTPA-PE-Gd conjugate. To chelate Gd3+ ions for MRI contrast,
phosphatidylethanolamine (PE) was conjugated with diethylenetriamine pentacetic acid (DTPA)
using a previously published protocol [50]. Triethylamine (60 µl) was added to egg
phosphatidylethanolamine (200 mg), which was dissolved in 8 ml of chloroform; the solution
was then added drop-wise to 1mM of DTPA anhydride solution (800 mg in 40 ml of
dimethylsulfoxide) and the mixture was stirred for 3 h under nitrogen atmosphere in an open
flask. The suspension was dialyzed overnight, using 2kDa molecular weight cutoff membranes
(Spectra/PorSpectrum Labs, CA), to eliminate free DTPA, and the resulting conjugate was
lyophilized. Gadolinium trichloride (37mg - equivalent to 40.0 mmol) dissolved in 0.2ml of
water was added drop wise to the DTPA-PE mixture (dissolved in 40ml DMSO) and stirred for 1
h, followed by dialysis with water using 2k membrane; the water was changed three times a day.
The final DTPA-PE-Gd mixture was lyophilized for 2 to 3 days. As free gadolinium is a known
toxin, being a heavy metal, and may contribute towards total gadolinium concentration, the
amount of free gadolinium in the complex was checked using the 200ul of 0.2mM Arsenazo dye.
Arsenazo III binds to metal ions forming an Arsenazo-metal ion complex which was
qualitatively analyzed by the color change. The final product was stored at -80ºC and was used
to prepare the formulations.
5.2.2 Formulation and characterization of Gd3+ labeled nanoemulsions for MR imaging

Gd\textsuperscript{3+} ions containing nanoemulsions were prepared by a high energy ultra-sonication method. From above Gd-DTPA-PE complex (0.5mmoles Gd\textsuperscript{3+}), Lipoid\textsuperscript{®} E80 (48 mg), tween 80 (8 mg) and stearylamine (8mg) were added to water (1.6 ml) and the mixture was stirred for 30 min to achieve complete dissolution of these excipients. Separately, 0.4 g of Flaxseed oil was taken in a glass vial. The two phases were then heated on a hot plate at 70ºC for 3-5 minutes. The aqueous phase was added to the oil phase and the mixture was sonicated at 21% amplitude and 50% duty cycle (Sonics and Materials Inc., Vibra Cell VC 505, Newtown, CT) for 10 min, resulting in the formation of the nanoemulsion (Figure 32). The mean particle size and zeta potential of the nanoemulsions droplets was measured as described in the section 3.2.1 with a 200 fold dilution of formulation with distilled water.

Magnetic resonance imaging (MRI) was used to determine the T1 relaxation times of the Gd- containing nanoemulsions. A magnetic pulse was sent through the imager, which decreased the relaxation time of the samples. According to the exponential decay function, the magnetization at T1 is 1/e, or approximately 1/3 less than the equilibrium magnetization. Hence, by extrapolating the time at this magnetization, it was possible to determine the T1 relaxation rate. Nanoemulsion was diluted into eppendorf tubes and run through a Bruker Biospin Bruker Biospec 7.0T /20-cm USR horizontal magnet. Magnevist solution which is used as a control was also diluted and run for \textit{in vitro} T1 values measurements.
Figure 3.2: Schematic representation of a nanoemulsion (NE) complexed with DTPA-PE-Gd3+.

A: Surface arrangement of phosphatidylcholine molecules with lipophilic tail embedded within the oily droplet while polar head groups positioning on the interface of the oil and water phase. B: Association of DTPA-PE-Gd3+ molecules with the oily droplet in analogous manner to phosphatidylcholine [50]

5.2.3 Use *in vivo* imaging to evaluate over time CNS distribution of formulations, following intranasal administration of the DTPA-PE-Gd3+ ion-containing nanoemulsion formulations in rats

To evaluate the uptake of the intranasal dosed formulations and to study distribution of NE we used Gd as a contrast agent. Sprague Dawley rats were administered DTPA-PE-Gd3+ ion-containing nanoemulsion formulations to rats at 0.02mmoles/kg (40ul of total nanoemulsion using the 50ul Hamilton syringe, 5ul volume administered in alternative nostrils and total volume administered in 20 minutes). Rats were placed in a head restrainer for imaging at various time points after the dosing. Pre-dosing images of the rat’s brain regions were also collected for comparison of T1 values.

**Study Protocol:** Adult, Female Sprague Dawley Rats were used in this study. Experiments were conducted using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, Massachusetts) and a 20-G/cm magnetic field gradient insert (ID = 12 cm)
capable of a 120-μs rise time (Bruker). Radiofrequency signals were sent and received with the quad-coil electronics built into the animal restrainer.

*Method of intranasal administration:* On test days, animals (n=6) were lightly anesthetized first for placement in the restrainer. Hamilton syringes (1ml) prefilled with the contrast agent containing formulations, or control, or Magnevist solution were attached to PE50 tubing and tubes were placed into the two adjacent nostrils of the rats housed with the isoflurane cone into the restrainer. Syringes were set up in the infusion pump to deliver 2.5ul volume at a time with the one minute time interval between dosing. Rats were finally positioned in the magnet and following scans were collected for each subject.

*Anatomical scans:* At the beginning of each imaging session, a high-resolution anatomical data set was collected using the RARE pulse sequence (20 slice; 1 mm; field of vision [FOV] 3.0 cm; 256 × 256; repetition time [TR] 2.5 sec; echo time [TE] 12.4 msec; NEX 6; 6.5-minute acquisition time).

*T1 Measurement:* Variable TR images were acquired using RARE pulse sequence (TE=12.5 and TR: 450, 800, 1400, 2200, 6000, msec.) Images were acquired with a field of view [FOV] 3 cm2, data matrix = 128x128x20 slices, thickness = 1 mm. T1 measurements were computed using Paravision 5.1 software (Bruker, Billerica, Massachusetts) by fitting absolute signal at particular TR.

Isoflurane was constantly supplied throughout the imaging session using a nose cone to maintain the respiratory rate between 40 to 60 breaths per minute. During image acquisition respiratory rate was monitored continuously over the entire imaging period using a small animal heating and monitoring system (SA Instruments, Stonybrook, NY). After the first baseline scan (pre-dose scan) of whole brain, rats were administered a total dose of 0.1mmoles/kg based on
body weight. After dosing is finished (within 30 to 40min), MR scan were commenced to capture post dosing time point of 30min, 60min and 90min.

5.3 RESULTS AND DISCUSSION

5.3.1 Synthesis and purification of phosphatidylethanolamine (PE) conjugated to diethylenetriamine pentacetic acid (DTPA), in order to chelate Gd3+ ions for enhancing MRI contrast

DTPA-PE-Gd complexed formed was found to be free of any Gadolinium ions when tested with arsenazo dye. These complex were further used in the nanoemulsions where the DTPA-PE-Gd complex was integrated into the nanoemulsion layer and Gd ion on the surface served as the contrast agent for the MR imaging studies in vivo.

5.3.2 Formulation and characterization of Gd3+ Nanoemulsions for MRI imaging

Particle size characterization showed the z-average of the particle distribution to be 342.4±21nm with a PDI of 0.20 and a surface charge of 26.5mV ±0.833vM. DTPA-PE-Gd3+ ion-containing nanoemulsions were also characterized using in vitro magnetic relaxivity. In-vitro T1 relaxation time measurements by MRI allowed for speculation on the contrast agent capabilities of the nanoemulsion formulations using parameters outlined in Figure 33. Figure 34 relates concentration of Gd3+ with the reciprocal of T1, a relation that results in a linear line with a slope of 6.58 sec-1 mmole-1. This slope is referred to as R1 and indicated a material’s relative contrast efficiency. R1 values of the NE-Gd were compared to Magnevist® solution which is commercially-marketed contrast agent and served here as the standard basis for comparison. It is
clear from Figure 34 that gadolinium loaded nanoemulsions significantly reduced the relaxation time relative to pure water and the higher slope of NE-Gd compared to Magnevist suggests that they might serve as comparable, if not better contrast agents, to solution of DTPA-Gd$^{3+}$ complex (Magnevist). Therefore, we conclude that this nanoemulsion system can serve as a strong contrast agent and we used these as contrast-enhancing agent to further study the uptake and distribution of formulation from nose to brain in rats.

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<tr>
<td>Recovery time [ms]</td>
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Figure 33: Outlined parameters used for capturing T1 weighted imaging for gadolinium-containing nanoemulsion

Figure 34: In vitro MRI relaxation rate plot of NE and Magnevist (R1= 1/T1) as function of concentration of DTPA-PE-Gd$^{3+}$. 
The $R_1$ value of $6.58 \text{ sec}^{-1} \text{ mmole}^{-1}$ for NE-Gd and for Magnevist standard $3.99 \text{ sec}^{-1} \text{ mmole}^{-1}$

5.3.3. **In vivo imaging to study the distribution of the nanoemulsion**

**Data Analysis and processing of images:** Each subject at different time point 0, 30min, 60min and 90min was registered to a 3D segmented and annotated rat brain atlas (Ekam Solutions LLC, Boston MA.). The alignment process was facilitated by an interactive graphic user interface. The affine registration involved translation, rotation, and scaling in all 3 dimensions independently. The matrices that transformed the subject’s anatomy to the atlas space were used to embed each slice within the atlas. All transformed pixel locations of the anatomy images were tagged with the segmented atlas regions, creating a fully segmented representation of each subject. T1 parameter values for each ROI was computed based on each segmented map (Figure 35)

![Figure 35(a) Brain atlas overlays of axial registration, (b) coronal view segmentation and (c) sagittal view segmentation](image-url)
% Change decrease in T1 values at each time point and in each major regions and specific regions was calculated at 30min, 60min and 90min time point as described:

\[
\text{% Change in T1 value = } \frac{(\text{T1 in region 1 at 0 min time point} - \text{T1 in region 1 at time point 30min}) \times 100}{\text{T1 in region 1 at 0 min time point}}
\]

Statistical student’s \( t \) tests were performed on % change of T1 value for major brain regions and specific 174 brain regions of each subject. T-test statistics using a 95% confidence level, 2-tailed distributions, and heteroscedastic variance assumptions were performed (highlighted in yellow), followed by T-test statistics using a 99% confidence level, 2-tailed distributions was also performed (highlighted in green).

Figure 36: Gd\textsuperscript{3+} ion containing nanoemulsion distribution at 25-30min time point compared to Magnevist and control in major regions of brain
Statistical test results shown in the row at the bottom of graph for comparison of NE-Gd and control.

MR imaging of the different major regions of brain following administration of NE-Gd intranasally resulted in a unique and widespread distribution of NE-Gd in brain as is evident by the significant decrease in T1 values in the major regions of brain (Figure 36). Uptake seems to be fast within 30min all the regions showed higher T1 value drop and most of the regions were found to be statistically different to control group except the cerebellum and amygdaloidal regions where although there was a higher change in T1 value, the results were not found to be significant.

When data was compared at 60min time point post dose, cerebrum, major area other, cranial nerves and ventral striatum showed the significant difference. Although other regions did show higher T1 value changes, they were not found to be significantly different. Results at later time point of 90min had similar profile with higher uptake observed with NE-Gd however results were not found to be significantly different (Figure 37 and Figure 38).

When data was analyzed for the 176 specific regions of brain, we found 22 brain regions out of 176 regions showing higher and significant uptake for the NE-Gd compared to control (Figure 39 and Figure 40). Magnevist distribution in brain was found to be lower than the NE-Gd distribution (as evident from the higher decrease in T1 changes due to NE-Gd) suggesting a possibility that nanoemulsion are being taken up by intracellular endocytosis process or through the trigeminal pathways where they could lead to higher uptake in different regions of brain. Further, nanoemulsion shows signal in different regions of brain for a longer time period then Magnevist which might be due to NE having higher residence time in nasal mucosa and as
residence time is directly proportional to nasal absorption of compounds we see higher and longer duration of uptake in certain regions of brain.

Figure 37: Nanoemulsion-Gd distribution at 55-60min time point compared to Magnevist and control in major regions of brain.

Magnevist® and saline used as a control. Statistical test results shown in the row at the bottom for comparison of NE-Gd and Control.
Figure 38: Highlighted major brain regions in an anterior to posterior overlay of rat brain atlas showing significant change in T1 values for Nanoemulsion-Gd compared to control or Magnevist® at 25-30min time point post dose.

Figure 39: Nanoemulsion-Gd distribution at 25-30min time point compared to Magnevist and control in specific regions of brain

Statistical t-test results shown in the row at the bottom for comparison of NE-Gd and Control.
5.4 CONCLUSIONS

The present study demonstrates that Gd$^{3+}$ ion containing nanoemulsion can bypass the blood-brain barrier to reach multiple sites within the brain as early as 30min after the intranasal administration. In some brain regions there is significant change in T1 values up to 10 to 15%. It is evident that some of the areas where higher T1 changes were observed are not in close proximity of olfactory bulbs, which suggests uptake of nanoemulsion through the trigeminal pathways. Furthermore, the distribution and uptake was found to be significantly different then the uptake of Magnevist aqueous solution. This could be due to low residence time of aqueous solutions into the nasal cavity and hence less chance for nasal absorption by the olfactory epithelium due to rapid clearance from mucociliary mechanism. We have demonstrated that intranasal application of NE-Gd results in rapid delivery to multiple areas of CNS which are known to be involved in the pathogenesis of neurodegenerative diseases.
CHAPTER 6

QUANTITATIVE EVALUATIONS AND COMPARISON OF DISTRIBUTION AND PHARMACOKINETICS UPON INTRANASAL AND INTRAVENOUS ADMINISTRATION OF CYCLOSPORINE IN RATS

6.1 INTRODUCTION

Biologics like peptides, proteins and oligonucleotides are too large and too hydrophilic to penetrate the BBB from the systemic circulation and would be rapidly degraded by gastrointestinal enzymes or the liver cytochromes, if taken orally. A non-invasive therapy would be desirable for patients particularly for diseases that require chronic dosing such as those related to neuro-inflammatory diseases like Alzheimer’s and Parkinson’s.

Nanocarriers may offer an improvement for nose-to-brain drug delivery since they are able to protect the encapsulated drug from biological and/or chemical degradation, and extracellular transport by P-gp efflux proteins. The small (nano) diameter will potentially allow nanoparticles to be transported transcellularly through olfactory neurons to the brain via the various endocytic pathways of sustentacular or neuronal cells in the olfactory membrane, as described above in Chapter 1. Further, nanoemulsion could potentially be able to overcome the mucus barrier due to their mucoadhesive properties. The potential role of the mucus layer in the transport of nanoparticles in epithelial tissues was illustrated by the study performed by Behrens group [51]. Evaluation of uptake of 213 nm PS nanoparticles into mucus producing cultured MTX cells was performed. Analysis of cells following 120min incubation with nanoparticles (using fluorescence spectroscopy) showed a 2-fold increase in the amount of internalized nanoparticles when the mucus layer of the cells was removed prior to the experiment.
This chapter is focused on evaluating the use of nanoemulsion for the nasal delivery of peptide specifically anti-inflammatory cyclosporine peptide. To quantitatively determine if intranasal administration resulted in detectable peptide delivery to different regions of brain we first developed and optimized a sample extraction protocol and LC-MS-MS method for bioanalysis of CSA. In order to determine whether the intranasal route of administration could bypass the blood-brain barrier, the intravenous routes of administration was also compared. We evaluated the distribution of the cyclosporine peptide in different regions of the brain when delivered as nanoemulsion versus solution form; the goal was to study the effect provided by the different excipients used in the formulations. Because volume is a major limitation for dosing intranasal, we first planned to prepare a high-loading formulation for in vivo dosing (Chapter 2). LC-MS was used to determine drug concentration over different time points (30 minutes, 1 h, 2 h, 4 h, and 6 h) in brain and blood, and the ratio of blood-to-brain concentrations was calculated, to study the targeting of drug to brain via the nasal route.

6.2 MATERIALS AND METHODS

The Strata®-X-CW 33u polymeric weak cation 30mg/ml SPE columns were purchased from Phenomenex Inc. (Torrance, CA). The solvents were purchased from Fisher Scientific (Fair Lawn, NJ). The LC/MS/MS analytics was carried out at using API5000 AB Sciex system at PhenoLogix, division of Phenomenex Inc. (Torrance, CA). Ascomycin IS was purchased from Sigma-Aldrich (St. Louis, MO). cyclosporine was purchased from Tocris biosciences (Minneapolis, MN)
6.2.1 Development of analytical method for quantification of cyclosporine peptide in biological matrices using Solid phase extraction and mass spectrometry

The main objective of this study was to determine blood and brain levels of CSA in rats upon intranasal delivery. As we planned on using the lower dose to study and compare the uptake, it was crucial to find the best sensitivity levels for detection of CSA in biological matrices – brain, blood, liver, kidney, and spleen.

1. Selection of SPE: To facilitate analysis by removing impurities and to maximize sample sensitivity by concentrating the analyte, we explored solid phase extraction technique (Figure 41) to accomplish these objectives. SPE offers various advantages:

- Eliminates otherwise co-eluted impurities or instrument-fouling particulates.
- Concentrated analytes and improves sensitivity (and reduces LOD and LOQ)
- Facilitates the rapid and efficient, simultaneous processing of multiple samples
- Provides higher extraction efficiencies, with quantitative recoveries of analyses and low levels of contaminants
- Provides consistent, reproducible results

The key element to any SPE product is the sorbent selection. The physiochemical properties of the sorbent and analyte determines extraction efficiency and the overall quality of the separation. As cyclosporine is neutral and contains mostly hydrophobic amino acids (CSA-logP 3.64), weak cation exchange sorbent was explored for retention. At extreme pH like 8.6 and in presence of high salt neutral analytes like CSA may be retained on the alkyl spacer arm via a reversed phase mechanism on a cation exchange sorbent. A weak cation-exchange functionalized polymeric sorbent was selected to allow complete retention of CSA, making 100% organic wash
conditions possible. A 100% organic wash was used to ensure that the maximum amounts of interferences are removed from the target compound (Table 9).

![General process outlining steps in Solid phase extraction (SPE) process](image)

**Figure 41**: General process outlining steps in Solid phase extraction (SPE) process

**Table 9**: Solid phase extraction steps for extraction of cyclosporine peptide from brain matrix

<table>
<thead>
<tr>
<th></th>
<th>Steps</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample Preparation</td>
<td>150 μL rat brain homogenate and 600 μL 50 mM ammonium bicarbonate (pH~8.6)</td>
</tr>
<tr>
<td>2</td>
<td>Condition Step</td>
<td>1 mL 100% methanol</td>
</tr>
<tr>
<td>3</td>
<td>Equilibration Step</td>
<td>1 ml of 50mM ammonium bicarbonate</td>
</tr>
<tr>
<td>4</td>
<td>Sample Load step</td>
<td>Load sample from step 1 onto a Strata-X CW SPE column</td>
</tr>
<tr>
<td>5</td>
<td>Wash 1</td>
<td>400ul of 50mM ammonium bicarbonate</td>
</tr>
<tr>
<td>6</td>
<td>Wash 2</td>
<td>400ul of 50% aqueous methanol</td>
</tr>
<tr>
<td>7</td>
<td>Dry</td>
<td>in centrifuge at high speed for 1-2min</td>
</tr>
<tr>
<td>8</td>
<td>Elute</td>
<td>2X400ul of Methanol</td>
</tr>
<tr>
<td>9</td>
<td>Solvent Evaporation</td>
<td>using solvent evaporator at 45-50C temperature for 3 hours</td>
</tr>
</tbody>
</table>
2. Recovery of cyclosporine peptide using SPE: To determine the recovery of cyclosporine peptide after SPE, several concentration were prepared by spiking brain homogenates with 1or 5mg/ml of CSA stock in methanol. These samples were subjected to SPE and after solvent evaporation samples were analyzed by LC/MS/MS using the conditions described in Table 10. Cyclo 1 transition was used as the primary quantitation transition. The other transition produced poor signal at the low pg/ml concentration. Standard curve were also prepared for cyclosporine in brain homogenates.

Table 10: LC/MS/MS method conditions for cyclosporine peptide

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument</td>
<td>AB Sciex API 5000 LC/MS/MS, ESI Pos Polarity Agilent 1260 UHPLC with binary pump, high pressure autosampler and column heater</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>A = 5mM Ammonium Formate in distilled water B = 5mM Ammonium Formate in methanol</td>
</tr>
<tr>
<td>Column</td>
<td>Kinetex C8, 50x2.1 mm, 2.6 um, Cat # 00B-4497-AN</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.6ml/min</td>
</tr>
<tr>
<td>Gradient</td>
<td>60 % B to 95% B in 2 min, hold for 1.5min at 95 % B</td>
</tr>
<tr>
<td>Injection volume</td>
<td>30ul</td>
</tr>
<tr>
<td>Temperature</td>
<td>60C</td>
</tr>
<tr>
<td>Detection method</td>
<td>Multiple Reactions Monitoring of the QqQ MS system</td>
</tr>
<tr>
<td>Ion Source</td>
<td>ESI, Positive Polarity</td>
</tr>
<tr>
<td>Ion Source Parameters</td>
<td>CUR: 25.00, GS1: 60.00, GS2: 45.00, IS: 4500.00, TEM: 400.00: ON, CAD: 10.00, DP 80.00, EP 10.00, CXP 13.00</td>
</tr>
<tr>
<td>Mass Transition ID</td>
<td>Q1, Da</td>
</tr>
<tr>
<td>Cyclo 1</td>
<td>1220.1</td>
</tr>
<tr>
<td>Cyclo 2</td>
<td>1220.1</td>
</tr>
<tr>
<td>Cyclo 3</td>
<td>1220.1</td>
</tr>
</tbody>
</table>
3. **Sample preparation for brain tissue bioanalysis:** The frozen whole brain was divided into three parts – Olfactory bulb, mid brain and hind brain regions to study uptake from rostral to caudal regions. Tissues were rinsed with sterile saline to remove surface blood and then homogenized in 0.5ml, 3ml and 2 ml volume of saline for olfactory, midbrain and hind brain tissue respectively. Tissue homogenization was performed on ice using probe solicitor for 1 to 3min. 100 to 200ul of the tissue homogenate was used for the extraction and elute after drying was reconstituted and was analyzed using LC-MS/MS.

4. **Sample preparation for whole blood CSA bioanalysis:** Aliquots of whole blood (150 uL) were combined with 50 uL of internal standard (200 ng/mL Ascomycin in 50:50 MeOH: H2O) and 200 uL 2.5% ZnSO$_4$ in distilled water. After briefly vortexing samples, 300ul of 100% methanol was added and samples were again vortexed. The samples were centrifuged at 14000 rpm for 5min to remove cell debris and entire supernatant was loaded into the SPE columns. SPE method for whole blood samples was similar to the brain samples except equilibration was performed with distilled water and washing was performed using water first followed by 50% methanol. Samples were eluted with 100% methanol followed by drying using solvent evaporator

5. **Sample preparation for peripheral tissue bioanalysis:** Liver, spleen and kidney tissues were homogenized in 5ml, 2ml, and 2ml saline respectively. Aliquots of 200ul were used to perform SPE using similar process like the whole blood sample preparation. Samples were eluted with 100% methanol (2x) followed by drying using solvent evaporation before analyzing on LC-MS/MS.
6.2.2 Evaluation of brain uptake and PK of cyclosporine nanoemulsion and compare to solution when delivered through intranasal versus intravenous route of administration

All animal procedures were performed in accordance with protocols reviewed and approved by the Northeastern University’s Institutional Animal Care & Use Committee. Sprague Dawley female rats, 200-225g of body weight, were purchased from Charles River Laboratories (Cambridge, MA). Rats were housed with a free access to food and water. A 12h light /12 h dark cycle was held to keep a normal circadian rhythm in the animals.

**Study # 1 CSA brain uptake after intranasal dosing comparing NE versus aqueous solution formulations:** In a first study, animals were separated into ten groups (n=4 each). The grouping was according to two criteria, type of formulation used intranasal aqueous solution which is similar formulation composition except without any flaxseed oil (S I.N.) versus intranasal nanoemulsion (NE I.N.). Animals were first anesthetized with a mixture of ketamine/xylazine, at a dose of 80 and 20 mg/kg, respectively, and were placed in a supine position with their noses at an upright 90° angle [52]. Preliminary studies demonstrated that bolus administration of volumes >5 μl /nostril resulted in respiratory distress. Therefore, the administered volume was limited to 5 μl increments, staggered every 1-2 min apart, alternating nares for a total of 25 μl per side for the 5mg/kg dosing. Administration was performed using a 20μl pipette tip. The tip was positioned at the opening of the nostril while the animal was placed on its back, and formulation gradually released in accordance with the animal inspiration. Following intranasal administration, the animals remained in a supine position, with their noses at a 45° angle for 60 min in order to maximize absorption through the nasal mucosa. Animals were returned to their home cages after recovery from anesthesia.
After pre-determined time 30min, 1, 2, 4 and 6 hours rats were sacrificed and blood was collected in EDTA treated tubes through cardiac puncture and organs were excised. Briefly, the rat was placed in a supine position, and an incision was made in the abdomen at the level of the xiphoid process just below the sternum. The diaphragm was then cut, and the heart was exposed by cutting along the bottom of the ribs and towards the front paws. Blood is collected using a 3ml syringe from the heart right atrium. Brain, olfactory bulbs, liver, kidney and spleen were collected, rinsed with distilled water and stored in -80°C until further peptide extraction was performed. The rats were administered with an equivalent dose of cyclosporine in solution form; however, due to loading limitation higher volume was delivered (approx. 100uL).

**Study #2 CSA brain uptake study by intravenous dosing:** To confirm if intranasal delivery is able to bypass BBB and to compare uptake of CSA rats were dosed at 5 mg/kg. Formulation were first diluted with saline to 5mg/ml and then injected as high concentration was viscous for injection and also low volume will lead to loss of formulation during IV injection. Rats were briefly sedated using isoflurane and injection of CSA solution or CSA NE was performed through the caudal vein of the rat tail. Rats were sacrificed as stated above and blood, brain, liver, kidney and spleen were collected for further CSA quantitation using SPE and LC-MS/MS method.

**CSA plasma and brain PK analysis:** Brain regions and whole blood analysis was performed using SPE sample extraction and LC-MS/MS quantitation method as detailed above. Brain tissue was dissected coronally and divided into three parts –brain front (olfactory), mid brain (BM) and hind brain (HB) as shown (Figure 42). For whole blood 200ul of aliquots were used for the SPE and quantitation. CSA pharmacokinetic parameters were determined using non-compartmental analysis with Phoenix® WinNonlin® v. 1.3 software. Area under the plasma
concentration-time curve from zero to infinity (AUC0-last) was calculated using the log-linear trapezoidal method.

**Figure 42:** Coronal sectioning of rat brain performed to evaluate the drug uptake in different regions of brain

*Biodistribution of CSA in highly perfused tissues from solution and Nanoemulsion formulation dosing:* Tissues from 4 hour time point were used for quantitation of CSA in peripheral tissues. For sample bioanalysis the frozen tissues were suspended in normal saline 5ml for liver, 3ml of spleen and kidney. Liver, spleen and kidney tissue were weighed and homogenized in 5ml, 2ml, and 2ml saline respectively at 5000 rpm for 3 minutes. SPE was performed using 200ul of homogenates. LC/MS/MS analysis of peptide was performed as previously described in section 6.2.2 and the data was reported as nanogram/gram of tissue.

**6.2.3 Statistical data analysis**

The statistical significance of all the data related to PK and distribution of CSA solution and nanoemulsion was compared and determined using the one-way ANOVA and Bonferroni’s post-test.
6.3 RESULTS AND DISCUSSION

6.3.1 Development of analytical method for quantification of cyclosporine peptide in biological matrices using solid phase extraction and mass spectrometry

As cyclosporine is a highly hydrophobic peptide, SPE sorbent like C8, C18 didn’t show good recovery even though the interferences were mostly purified. SPE-weak cation exchange sorbent showed best recovery with good accuracy at different spike concentration. The API5000 LC/MS/MS system provided a linear standard curve in the concentration range of 0.1-50 ng/ml (Figure 45A), which was used for samples quantitation. The LOQ was set at 100pg/ml which for a 30 all injection volume, translates to 3 pg of peptide. Spiked standards in whole brain sample matrix (SPE fraction) showed clean MS chromatograms (Figure 43-44) with limited interference from biological matrix and hence, improved the quality of data. For whole blood samples 1ng/ml to 200ng/ml range showed good linearity (Figure 45B). Similarly, for peripheral tissues analysis, we established the linear range first using the blood extraction procedure and obtained good linearity and accuracy in liver, spleen, kidney, heart and lung matrix (Figure 47).
Figure 43: Spike recovery for cyclosporine in brain samples showing good accuracy.

Figure 44: A representative chromatogram of 100 pg/mL spiked rat brain extract
Figure 45: Representative calibration curve for cyclosporine, in brain homogenates ranging from concentration of 0.1ng/ml to 50ng/ml (A) and in rat whole blood ranging from concentration of 1ng/ml to 200ng/ml showing high correlation factor.

<table>
<thead>
<tr>
<th></th>
<th>Analyte Conc. (ng/mL)</th>
<th>Calculated Conc. (ng/mL)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCL</td>
<td>48</td>
<td>52.9</td>
<td>110</td>
</tr>
<tr>
<td>QCM</td>
<td>640</td>
<td>692</td>
<td>108</td>
</tr>
<tr>
<td>QCH</td>
<td>3200</td>
<td>3150</td>
<td>98.5</td>
</tr>
</tbody>
</table>

Figure 46: A Representative calibration curve for cyclosporine in rat liver ranging from concentration of 16 ng/ml to 4000 ng/ml.

We established solid-phase extraction (SPE) technique using a weak cationic column, for isolation of CSA peptide analyte from the in vivo samples. API 5000 LC/MS/MS system from AB Sciex was used for analyses of PK and distribution samples. Results showed linear fit for the calibration curves prepared with brain or blood biological matrix. CSA showed the best recovery.
almost 100% from weak cation exchange polymeric SPE sorbent. Protein precipitation was not found to be successful during the initial sample extraction procedures. However, protein precipitation procedure was successful at higher concentration such as 50ng/mL or higher in brain tissue matrix.

6.3.2 Evaluation of brain uptake and PK of cyclosporine nanoemulsion and compare to solution when delivered through intranasal versus intravenous route of administration

Following intranasal administration, high levels of CSA appeared in olfactory bulbs and in the mid brain regions (Figure 47). Olfactory bulb showed the Tmax at early time point of 60min and it was similar found to be similar for hind brain and blood. Interestingly, NE I.N. showed later Tmax of 4 hours in mid brain which could be result of higher residence time of the NE in nasal mucosa compared to solution form or IV route of administration. Although the levels were found to be highest at all-time points using intranasal administration via nanoemulsion(NE I.N.), compared with both intranasal administration of aqueous solution(S I.N.) and intravenous administration at the same dose of nanoemulsion(NE IV) or solution form(S IV), the results were not statistically significant (Figure 48). This could be due to high variability in brain levels.
Figure 47: The mean ng/gm concentration–time curves of CSA in different regions of rat brain after intranasal and intravenous administration.

cyclosporine administered as CSA-NE and CSA-Solution at the dose of 5 mg/kg. Results show NE I.N. leads to higher exposure in every part of the brain (especially BM) than any other strategy – (NE IV, S-IV, and S-I.N.).
Figure 48: The mean ng/gm brain concentration–time curves of CSA in rats after intranasal and intravenous administration of cyclosporine administered as CSA-NE and CSA-Solution at the dose of 5 mg/kg. Results show NE I.N. leads to higher exposure in brain especially at later time of 4 hour than any other strategy – (NE IV, S IV, and S I.N.).

*Plasma pharmacokinetic (PK) analysis of CSA peptide following I.N. versus IV administration from solution or nanoemulsion formulation:* When comparing the plasma concentration (Cp)–time data for CSA solution and nanoemulsion after I.N. or IV administration at 5mg/kg (Figure 49), a significant decrease in levels of CSA in blood was seen with NE I.N. and S I.N. when compared to NE IV or S IV. Intranasal route of delivery leads to less exposure to blood overall whether the delivery is with particulate system like NE or simple solution. The statistical significance of the results was determined using one-way ANOVA and Turkey’s Multiple Comparison Test. Statistical results when comparing routes and also comparing formulation strategy shows that blood levels are significantly lower at 60, 120 and 240 min after S I.N. administration, and 240 min after NE I.N. administration, when compared to S IV. Further, statistically significant lowering of CSA uptake in whole blood observed at 240min time point for the NE I.N. delivery versus S IV delivery. These results shows that nanoemulsion are preferentially being endocytosed by nasal olfactory epithelium cells as compared to the
respiratory epithelium cells and less of the particles are being able to distribute to the systemic circulation due to this reason. Brain to blood concentration ratios were calculated at each time point and it was found that ratio was higher at all-time points with NE I.N. (Figure 50). When comparing ratios of routes/formulations, statistically significant difference is obtained only between ratios at 60 min and 120 min (ANOVA, Bonferroni’s post-test).

Figure 49: The mean blood concentration–time curves of cyclosporine in rats after intranasal and intravenous administration

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Blood 60</th>
<th>Blood 120</th>
<th>Blood 240</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE I.N. versus S I.N.</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>NE I.N. versus NE IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>NE I.N. versus S IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>S I.N. versus NE IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>S I.N. versus S IV</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>NE IV versus S IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>
Results shows I.N. route reduces blood exposure when compared to IV route and furthermore NE-I.N. shows higher brain targeting compared to S-I.N..

The area under the blood concentration–time curve AUCblood value and brain concentration–time curve AUCbrain value for each of the different regions were calculated using the trapezoidal rule (Figure 51). AUC for OB and AUC of MB were found to be statistically different and higher for NE I.N. compared other strategies.
Figure 51: Comparison of AUCLast for I.N. and IV route of delivery for both CSA nanoemulsion and CSA- solution

*NE I.N. leads to higher exposure of every brain sub-part, when compared to others, with low blood exposure (comparable to S I.N.). S I.N. leads to similar brain exposure as S IV, but to lower blood exposure. NE IV induces very low brain exposure, and high blood exposure, although blood exposure is lower than for S IV. The statistical significance of the results was determined using one-way ANOVA and Bonferroni’s post-test.*

<table>
<thead>
<tr>
<th>AUCLast</th>
<th>BF</th>
<th>BM</th>
<th>BB</th>
<th>Brain</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE I.N. versus NE IV</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.01</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>NE I.N. versus S I.N.</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>NE I.N. versus S IV</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>NE IV versus S I.N.</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>NE IV versus S IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>S I.N. versus S IV</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

*Brain Targeting Effect:* The degree of CSA targeting to brain after intranasal administration can be evaluated by the drug targeting efficiency, which can be described as the ratio of the value of AUCLast\textsubscript{brain}/AUCLast\textsubscript{blood} following Intranasal or Intravenous administration. The higher the targeting efficiency, the further degree of CSA targeting to brain can be expected after the respective route of delivery. We observe a measurable degree of CSA targeting to brain following intranasal administration using NE formulation strategy. Ratio was found to be statistically (Figure 52).
Targeting Efficiency = \( \frac{\text{AUC}_{\text{last of Brain}}}{\text{AUC}_{\text{last of Blood}}} \)

Figure 52: Comparison of targeting efficiency of I.N. and IV route of delivery for both CSA-nanoemulsion and CSA-solution.

Figure shows that the ratio between brain and blood exposure is in favor of I.N. administration, and that NE further enhances this ratio. NE I.N. induces a ratio significantly higher than any other treatment strategy. The statistical significance of the results was determined using one-way ANOVA and Bonferroni’s post-test.

<table>
<thead>
<tr>
<th>AUClast</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE I.N. versus NE IV</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>NE I.N. versus S I.N.</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>NE I.N. versus S IV</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>NE IV versus S I.N.</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>NE IV versus S IV</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>S I.N. versus S IV</td>
<td>( P &gt; 0.05 )</td>
</tr>
</tbody>
</table>

Absolute bioavailability of cyclosporine drug was calculated for NE dosed intranasal and Solution-CSA dosed intranasal. When comparing the fold increase in bioavailability we found NE dosed intranasally improved bioavailability almost 20x fold compared to CSA-solution dosed intranasally (Table 11).
Table 11: Bioavailability of cyclosporine drug upon administration of CSA-NE and CSA-solution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC last</th>
<th>%F</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE I.N.</td>
<td>134528.7</td>
<td>2.8</td>
</tr>
<tr>
<td>NE IV</td>
<td>4806.3</td>
<td></td>
</tr>
<tr>
<td>S I.N.</td>
<td>26842.6</td>
<td>0.1</td>
</tr>
<tr>
<td>S IV</td>
<td>20065.8</td>
<td></td>
</tr>
</tbody>
</table>

**CSA biodistribution in highly perfused tissues following I.N. versus I.V. route of administration of solution or nanoemulsion formulation:** Examination of peripheral tissues for the distribution of CSA was performed to check if brain targeting obtained with CSA-NE I.N. was brain-specific, or if non-targeted organs were also more exposed to the drug. Intranasal administration resulted in overall considerably lower exposure of peripheral organs to CSA when compared to IV administration (**Figure 53**). CSA-NE formulation further reduced the non-targeted organ exposure, especially for liver, although no difference was found significant. Interestingly, NE formulation induced lower concentrations versus solution in liver, kidney and spleen after IV administration, while lung and heart appeared more exposed.

![Figure 53: Comparison of cyclosporine distribution 4-hour post administration in peripheral tissues from I.N. and IV route of delivery for both CSA- nanoemulsion and CSA-solution.](image)
6.4 CONCLUSIONS

Our findings from PK and distribution of CSA reveals that a significantly high quantity of cyclosporine can be effectively delivered to the different regions of brain by intranasal administration of formulated nanoemulsion compared to simple solution form. It is likely that protection of peptide inside a nanoparticle system and the longer residence time in the nasal cavity due to their mucoadhesive nature, resistance to P-gp efflux is the reasons for the promotion of the uptake of the peptide over and above that of simple peptide solution. Nanoemulsion offered high exposure in mid brain at later time point which highlights the advantage of using mucoadhesive systems for intranasal delivery. Another advantage that was highlighted by the delivery of nanoemulsion intranasally is the overall less systemic exposure and peripheral tissue exposure. We believe these nanoemulsions are preferentially being endocytosed by the olfactory epithelium cells than the respiratory epithelium cells due to the electrostatic interaction possible between the mucus and cell surfaces. Mucus contains salic acid carries a net negative charge and as a consequence, mucin and nanoemulsion can bind strongly which lead to overall higher residence time in the olfactory epithelium part of the nasal cavity. Furthermore, these systems are biocompatible and biodegradable as they are prepared using flaxseed oil, a rich source of omega-3 fatty acid, which plays a key role in brain function specifically in cognitive and behavioral function in CNS.
This combination of mucoadhesive and biocompatibility has led to a consideration of the use of nanoemulsion for the delivery of different peptides via nose to brain. Further, surface modification of these nanoparticles could be achieved for targeted CNS delivery of a number of different biologics drugs using the same ‘platform’ delivery system which has known and well characterized biophysical properties and mechanism(s) of transit into the CNS.
CHAPTER 7

EVALUATION OF BRAIN AND PLASMA EXPOSURE FOR SIRNA DELIVERED IN NANOEMULSION IN COMPARISON TO SIRNA DELIVERED IN AQUEOUS SOLUTION FORMULATION

7.1 INTRODUCTION

neuro-inflammation is a common denominator in various neurodegenerative diseases, such as Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), Huntington’s disease, Parkinson's disease, head trauma, epilepsy, and stroke [53]. These disorders are devastating and expensive, with an annual cost currently exceeding several hundred billion dollars in the United States alone, and current palliative treatments are inadequate. Although the current research has focused on understanding the mechanism(s) and pathological pathways underlying the various NDDs and their associated drug targets, delivery of the therapeutic molecules at the targeted site remains a major issue [54]. RNAi technology for targeted modulation of gene expression in CNS has potential in neuro-inflammation. RNAi therapy utilizes short interfering RNA (siRNA) duplexes, usually composed of 20-25 nucleotides, and is involved in post-translational gene-silencing mechanism. When delivered efficiently to the CNS and to the cytosol of the microglial cells, the antisense strand of the siRNA will interact with RNA inducing silencing complex (RISC) and breakdown the complementary mRNA sequence with the action of the argonaute enzymes. Ultimately this approach will block the expression of specific genes, such as TNF overexpressed in neuro-inflammation [55]. The exquisite selectivity and high potency of appropriately designed siRNA sequences affords an excellent therapeutic opportunity for chronic treatment of neuro-inflammation without the associated toxicities.
However, for translating RNAi from an experimental approach to a clinical-viable therapeutic strategy that can benefit patients, there is a critical need for a safe and effective delivery system that can package the labile payload, afford stability during transport, permeability through tissue and cell membrane barriers, as well as cytosolic bioavailability for efficient gene silencing. For diseases of the CNS, opportunity for systemic RNAi therapy is further dampened by the formidable BBB, which limits transport of up 98% of small molecule therapeutics and 100% of large molecular weight hydrophilic compounds, such as proteins and nucleic acid constructs.

In order to overcome these obstacles, to this end we formulated siRNA against TNFα, in a cationic nanoemulsion of omega-3 fatty acids and investigated the delivery through intranasal route to enhance CNS delivery of siRNA. TNF-α is a potent pro-inflammatory cytokine that is a member of the TNF superfamily of ligands. However, under chronic inflammatory conditions, TNFα facilitates progression of the disease including, gliosis, demyelination, and BBB deterioration. The hypothesis for this approach is that siRNA complexed with cationic lipid in a nanoemulsion will be protected from enzymatic degradation in the nasal mucosa, further emulsion will provide higher mucosal residence time for uptake and lastly intranasal route of administration will provide direct access to brain by bypassing the BBB.

Successful delivery of small interfering RNA (siRNA) to the CNS has been demonstrated following intranasal delivery by few researchers by using labelled siRNA. FITC-labeled transfection control siRNA was detected in the olfactory bulb 12 h after I.N. administration [56]. In another study, delivery of fluorescently labeled siRNA from the nasal cavity to the olfactory bulbs via the olfactory nerve pathway was examined. siRNA was observed along the length of the olfactory nerve bundles, from the olfactory mucosa of the nasal cavity to the anterior regions
of the olfactory bulbs. In the olfactory bulbs, siRNA was observed in the olfactory nerve, glomerular and mitral cell layers (Figure 54). These results demonstrate a role of the olfactory nerve pathway in targeting siRNA to the olfactory bulbs [57].

![Image](image_url)

Figure 54: Thirty minutes following the intranasal administration of fluorescently labeled siRNA (red) to mice depicted in picture. 

*Label was observed in the olfactory (OE) and lamina propria (LP). (A, C) Image consists of a 63 μm z-stack of 42 confocal images. (A, B) Scale bar = 30 μm, (C) scale bar = 100 μm. * = surface of the ONB. 120 μg of labeled siRNA was administered. Red = DyLight647 (siRNA), green = fluorescein labeled Ulex agglutinin, blue = DAPI [57].*
Additional investigations are required to assess the distribution of intranasally delivered non-labelled, therapeutic siRNA to brain. In this study we have utilized stem-loop PCR quantification method to detect and quantify siRNA in vivo. This method should be able to detect non-modified siRNA in tissues and hence will not be biased by the label or the accumulation of metabolites of label siRNA.

We have previously shown that TNF-α siRNA was successfully complexed in omega-3 polyunsaturated fatty acid based cationic NE with at least 1mg/ml loading and further we scaled formulation to achieve upto 3mg/ml loading. When NE systems were evaluated for uptake in vitro we found higher levels of CY3 label siRNA using confocal and FACS analysis. We utilized the J774 cells as surrogate macrophages to microglia for studying induction and suppression of cytokines markers during cell stress by LPS toxin. When tested efficiency of siRNA encapsulated nanoemulsion in J774 macrophages in vitro, we found that silencing efficiency of SNE (siRNA nanoemulsion) was higher than the siRNA silencing efficiency was comparable to Lipofectamine transfected cells at least at 12 hour time point. Furthermore, the duration of effect observed with SNE siRNA was found to be greater (24hours) than the duration of effect using siRNA Lipofectamine transfection.

Based on these preliminary in vitro finding, showing promising results we proceeded with cationic based SNE to further study the uptake of siRNA in brain. The major goal of this study (Aim 7) was to determine if intranasal administration of cationic nanoemulsion formulations can successfully achieve higher uptake in brain compared to non-encapsulated siRNA delivered as solution.
7.2 MATERIALS AND METHODS

7.2.1 Chemicals and reagents

AgPath-ID One step RT-PCR kit and TaqMan gene expression assay was purchased from Invitrogen (Carlsbad, CA) to perform PCR. Primer and ligation oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). All PCR were performed in the ABI7500 (Applied Biosystems, Foster City, CA) and RT reactions were performed in the Bio-Rad thermocycler. Probes were purchased from Applied Biosystems, Inc. (Foster City, CA) and were designed using Primer Express software (Applied Biosystems). The TNF-\( \alpha \) targeted siRNA sequence previously published [58] and knockdown shown in mouse model was obtained from Anylnam (Cambridge, MA) as gift. The target TNF alpha gene siRNA target has the following strand sequences:

TNF-\( \alpha \) S: ucuucuGucuAcuGAAcuudTsdT

TNF-\( \alpha \) AS: AAGUUcAGuAGAcAGAAGAdTsdT

Lower case letters indicate bases with a 2’OMe backbone modification; all sequences are represented in 5’-3’ direction. S stands for the sense (passenger) strand, AS stands for antisense (guide) strand.

7.2.2 Intranasal siRNA delivery

All animal experiments were carried out in accordance with Northeastern University, Institutional Animal Care and Use Committee (NU-IACUC). Rats were anesthetized with an intraperitoneal injection of ketamine/xylazine hydrochloride injection. Body temperatures were
maintained at 37 °C using a heat lamp. The animals were randomly divided into five groups; animals in the first group were administered TNF-α siRNA(STNF-6hr) in saline solution and scarified after 6 hour after first dose administration, those in the second group were administered TNF-α siRNA(STNF-24hr), and animals in the third and fourth group were administered SNE(siRNA nanoemulsion) and fifth group was administered vehicle (PBS).

The nasal administration procedure was carried out as previously described where 5ul volume of solution or formulation was administered in alternating manner with a 2min interval between applications. After the first administration second dosing was performed under isoflurane to minimize the anesthesia overdose. SiRNA Nanoemulsion (SNE) and siRNA solution control in saline was injected every 2hour for 6hour and for 24 hour dosing, formulation was injected 2times on day 1 and day 2 according to the protocol below (Figure 55B).

TNF-α silencing siRNA duplex was encapsulated in DOTAP nanoemulsion as described before in Chapter 2 section 2.2.4. Briefly, DOTAP film was prepared first and siRNA was precomplexed with DOTAP, following which oil phase was added to the complexes mixture and then water phase containing lipoid E80 and tween 80 was added slowly to the above mixture. Mixture was finally homogenized and briefly sonicated using probe sonication to achieve final size of 200-300nm. As, higher concentration of siRNA loaded nanoemulsion was required due to limited volume that can be delivered in vivo in rats, formulations were scaled up to load up to 3.8mg/ml siRNA. Formulations were characterized for particle size, zeta potential and encapsulation before dosing in vivo.
7.2.3. siRNA quantitation in brain tissues and plasma

After 30 min following the last injection, blood samples and whole brain were collected. Plasma was separated from whole blood after centrifuging at 2000g for 10 min at 4°C. The brain tissue was then homogenized using Qiagen Tissue Lyser (Qiagen, Germantown, MD) to prepare tissue lysates. The homogenized tissue lysates were subsequently diluted at 1:500 to prepare dilute samples. Using the appropriate reverse primer, anti-primer and the tissue lysate, the annealing step was run initially followed by RT-PCR. The TNF-α siRNA sequence, reverse primer, forward primer, and an anti-primer sequence are listed below.

Reverse: GGAAGCGCGCAAGGCGTAA

Forward: /56-FAM/ACTCCCTCCCTCGATTTAAGTTTCAGTAGACA

Anti-primer: AAATCGAGGGAGGGAG/3BHQ_1/

As a first step, the siRNA was denatured and annealed to the RT primer (6 μl diluted siRNA and 18 μl reverse primer, 100 nM). siRNA was denatured by incubating at 95 °C for 5 min. Primers were then annealed by 2 min incubation at 80, 70, 60 and 45 °C with 4 °C hold. Then the reverse transcription reaction was carried out as follows. A master mix was made by mixing the following components: RT-PCR buffer (6.25 μl), forward primer (10 μM, 0.12 μl), reverse primer (10 μM, 0.12 μl), antiprimer (100 μM, 0.12 μl), 25 × RT-PCR enzyme (0.5 μl) and water (1.5 μl). A total of 17 μl of this master mix was then mixed with 7 μl of sample and ran the PCR at the following listed conditions: 50 °C (10 min), 95 °C (10 min), 40 cycles, 95 °C (15 s), 45 °C (60 s). The amplified siRNAs were finally detected and quantitated by running a standard curve using lysate from untreated vehicle rat tissue or plasma and spiked with known
siRNA concentrations ranging from 2000 ng/ml to 0.002 ng/ml. For rat plasma, samples were first diluted 1000 fold with PBS and quantitation was performed similar to the brain samples.

7.2.4. Statistical data analysis

Statistical analysis was performed using the two tailed unpaired t-test to compare the mean values±standard errors and to determine if there was any significant difference between delivery system; p values of 0.05 were considered to be statistically significant.

7.3 RESULTS AND DISCUSSION

7.3.1 siRNA quantitation in brain tissues and plasma using PCR method

For the siRNA quantification study, the TNF-α was encapsulated in identical DOTAP nanoemulsion at higher concentration of ~3.8mg/ml. Nanoemulsion showed particle size of 350nm and zeta potential of +42mV. Formulation were also analyzed for % encapsulation using sybr green dye and it was found that almost 100% encapsulation was achieved (Figure. 55A). Since substantia nigra in brain is our major target, we selected mid brain region to study the uptake of therapeutic siRNA. As intranasal delivery could lead to distribution on plasma, we assessed siRNA uptake in plasma as well. After three times dosing of siRNA in a day for a total of 3mg/kg doses through the nasal route, blood and tissue samples were collected for siRNA quantitation at 6 h and 24 h post first administered dose (Figure. 55B).

For the accurate siRNA quantitation, the anti-primer quenching based real time PCR method was utilized, as described in the experimental section [59, 60]. In this method, a fluorescently labeled PCR primer was designed to anneal to the template RNA and to a universal anti-primer. Following the initial PCR, the temperature is lowered to allow the anti-primer to
bind to the unincorporated primer to quench its fluorescence. Since this will not bind to the double stranded PCR product, there will be an increased fluorescent signal detected.

A.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>N/P Ratio</th>
<th>Z-average(nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>% Complexes</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>032214 SNE-TNF</td>
<td>4.86</td>
<td>353.3 ±7.69</td>
<td>0.295</td>
<td>42.2±3.3</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

B.

Figure 55: TNF-α loaded nanoemulsion characterization and study design

DOTAP-siRNA nanoemulsion (SNE) were prepared and the particle size, charge and % complexation and total quantitation was performed (A). SNE and siRNA in saline was used in the distribution study to determine uptake in brain and plasma. The study was designed to give a multiple dose of 3.0mg/kg siRNA dose with n=3/group animals (B).
Figure 56: Standard curves of TNF-α siRNA prepared using stem-loop assay in brain tissue (A) and plasma (B).

*Ct values of standard curves in brain ranging from 2000 ng/ml to 0.02 ng/ml and in plasma ranging from 2000 ng/ml to 0.002 ng/ml (plasma).*
7.3.2 Brain and plasma uptake results comparing nanoemulsion intranasal delivery to aqueous solution formulations

The PCR quantitation method employed for tissue and plasma siRNA quantitation showed good linearity for the standard curve prepared in respected biological samples (Figure 56A and 56B). The TNF-α siRNA was quantitated in mid brain region (Figure 57A) and the % input dose per brain region was calculated based on the starting siRNA dose. TNF-α siRNA delivered intranasally in saline solution showed 0.2% injected dose at 6 hour time point followed by a reduced 0.12% injected dose at later time point of 24hour. SNE (siRNA nanoemulsion) showed higher 1.38% injected dose in the mid brain region at 6 hour time point after a dose of 3mg/kg. Nanoemulsion also showed reduced, 0.28% of the injected dose at later time point of 24hour time point in brain. Even though the clearance observed for nanoemulsion was found to be similar to siRNA dosed in saline solution, the exposure of the mid brain region was found to be significantly higher (p<0.05) compared to siRNA in solution (STNF) at 6 hour time point. (Figure 57B) There was higher exposure from nanoemulsion at later time point as well, but the difference was not found to be significant. It could be due to the less number of animals used here n=3. Interestingly, SNE at 24 hour time point was able to provide exposure similar to the siRNA solution at 6 hour time which could be potentially due to the longer residence time and higher permeation through the nasal mucosa.
Figure 57: A representative region of brain used for the comparison of brain exposure by multiple dosing of siRNA through the nasal route (A). Graph shows the % injected dose in brain comparison for siRNA dosed in solution (STNF) versus siRNA dosed in nanoemulsion (SNE) formulation.

Overall the exposure in plasma was found to be lower than the exposure in brain with both the strategies delivered intranasal (Figure 58). SiRNA solution showed only 0.06% injected dose compared to siRNA nanoemulsion which was found to be 0.17% of the injected dose at 6 hour time point. At the later time point of 24 hour, siRNA solution delivered only 0.02% injected dose whereas siRNA nanoemulsion delivered 0.45% injected dose which was found to be
statistically significantly (p<0.005). It seems that plasma exposure overtime seems to be increasing with the nanoemulsion which could be due to the fact that emulsion due to its higher residence time in mucosa is being able to permeate though the nasal mucosa slowly and hence prolonged increase in exposure.

![Graph showing TNF-alpha levels in plasma after 3 MPK multiple dose](image)

**Figure 58**: Graph shows comparison for the % injected dose in plasma following siRNA dosed in solution (STNF) versus siRNA dosed in nanoemulsion (SNE) formulation.

Further, brain targeting ratio was calculated to assess and compare the advantage of both strategies delivered intranasal (Figure 59). The ratio is calculated based on the concentration in plasma and brain at each time point. Brain targeting ratio of siRNA was found to be almost 2 fold higher for SNE (nanoemulsion) compared to STNF (Solution) at early time point of 6 hour. At later time point of 24hr, the ratio for solution was found to be higher compared to nanoemulsion although it was lower than the ratio observed at early time point.
7.4 CONCLUSIONS

A sensitive RT-PCR based quantitative method was utilized to determine the real therapeutic siRNA amounts in brain and plasma. When comparing the siRNA uptake through the intranasal delivery it seems like overall higher brain uptake is possible compared to plasma. Out of the two strategy compared for brain targeting, nanoemulsion achieved higher levels in brain both at early time point and later time point when compared to siRNA solution as indicated by % injected dose in mid brain. Overall exposure in plasma was also found to be higher for nanoemulsion but more interestingly the uptake seems to be increasing over time when compared to solution of siRNA. The current findings demonstrate the utility of nanoemulsion delivery systems for enhanced delivery to target brain via intranasal route. Additional investigations will be performed to explore the capacity of the delivered siRNA to silence gene expression in CNS.

Figure 59: Plot showing the brain targeting ratio comparing the targeting efficiency of siRNA using either solution versus nanoemulsion
CHAPTER 8

ESTABLISHMENT AND CHARACTERIZATION OF AN LPS-INDUCED NEURON-INFLAMMATION RAT MODEL TO EXAMINE THE THERAPEUTIC EFFICACY OF INTRANASALLY DELIVERED FORMULATIONS FOR CYCLOSPORINE AND TNF-ALPHA SILENCING SIRNA

8.1 INTRODUCTION

Although the driving force behind the progressive neuronal loss underlying neurodegenerative diseases such as Parkinson’s, Alzheimer’s diseases, amyotrophic lateral sclerosis, and the AIDS dementia complex has remained elusive, recent evidence has suggested that chronic neuro-inflammation may be a key player in the degenerative process [61-64]. McGeer et al. first provided evidence for the role of inflammatory processes associated with PD where he demonstrated the presence of activated microglia in the substantia nigra of patients at postmortem. Initially the glial reaction observed in neurodegenerative disorders was generally considered to be simply a consequence of nerve cell death. However, recent evidence suggests the contrary, glial reaction might be also involved in the evolution of the disease. This is supported by several works describing different kinds of inflammatory features present in Parkinsonian brains: a dramatic proliferation of reactive amoeboid macrophages and microglia in the substantia nigra (SN; [61, 65, 66], along with the increase in glial cells expressing different pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ [67] (Figure 60). Neurodegeneration in AD occurs as a result of accumulation of aggregated amyloid plaques in the brain, which steadily increase with age. Along with increase in amyloid plaque accumulation, the AD brain also exhibits increased levels of pro-inflammatory cytokines which function as stimulators of neuro-inflammation.
Due to the central role of inflammation in various diseases, the need for purely inflammation-driven animal’s model has emerged. An in vivo LPS-induced PD model was developed by Castano et al., [68]. Subsequently, LPS induced PD model has been widely accepted and used for understanding pathogenesis of PD and for testing various anti-inflammatory treatment therapies for targeting neuro-inflammation. In this study, we first established the in vivo LPS induced neuro-inflammation model in rats by studying the dose dependent and time dependent effect of LPS on the stimulation of various cytokines in substantia nigra region of brain using intranigral microinjection into brain.

Although combination of various factors is thought to contribute to the neurodegenerative processes both in cell culture systems and animal models, the precise mechanisms of action remain to be elucidated. In order to evaluate the possible protective effects of anti-inflammatory compounds (cyclosporine and TNF-alpha) in LPS-induced degeneration of nigral dopaminergic neurones, we carried out two different kinds of studies:

(i) study the potential effect on various pro-inflammatory cytokines upon intranasal administration and (ii) further we tested the therapeutic effect at cellular levels by studying the neuroprotective role against microgliosis, astrogliosis and dopaminergic neurons.

After establishing the model, we have evaluated the therapeutic effect of cyclosporine and TNF-alpha siRNA individually and in combination through intranasal delivery.
Figure 60: Nuclear receptors and neuro-inflammation-induced dopaminergic neuronal damage.
(a) Schematic representation of the effects of inflammatory mediators released from activated microglia and astrocytes on dopaminergic neurons, and the consequent precipitating effects of oxidative species released from dying dopaminergic neurons on microglial activation. (b) The possible effects of the synthetic agonists like cyclosporine and TNF-alpha siRNA on damaged dopaminergic neurons, activated microglia, astrocytes, and T lymphocytes (Picture modified from [69]).

8.2 MATERIAL AND METHODS

Cyclosporine was purchased from Torcris biosciences. TNF-alpha gene silencing siRNA was a generous gift from Anylnam Pharmaceuticals (Cambridge, MA). All the primers and PCR reagent purchased from Invitrogen. LPS (Escherichia coli 0111:B4) and endotoxin-tested and sterile PBS were purchased from Sigma (St. Louis, MO) Harvard Infusion pump was generously provided by Dr. Barbara Calderone’s lab at the Harvard Neuro Discovery Center. Stereotaxic apparatus was borrowed from Dr. Craig Ferris lab in the Center for Translational Neuro-Imaging at Northeastern University.
8.2.1 Establishment of an LPS induced rat neuro-inflammation model

Firstly, we evaluated the time dependence and dose dependence effect of ug LPS injection in SN region of brain.

8.2.1.1 Surgical microinjection of LPS in the substantia nigra

Female Sprague Dawley rats (210–230 g) were obtained from Charles River Laboratories (Raleigh, NC) and kept on a 12-h light/dark cycle with ad libitum access to food and water. Rats were acclimated to their environment for 2 days before the experiments. Sterile surgical equipment and aseptic techniques were used for all procedures in compliance with Northeastern University IACUC policy and the approved animal protocol. Rats were deeply anesthetized first using Isoflurane. The rat’s head was shaved and swabbed with 70% isopropyl alcohol and betadine. Rats were then placed in the stereotaxic instrument. Body temperature was maintained throughout the procedure at 38 °C using a heating pad (Fintronics). A sterile scalpel was used to create a 1-2 cm rostral to caudal incision on the scalp, and to expose the bregma. Tissue overlying the suture lines was scraped away and the skull was dried using a dryer. A drill (Dremel®) was then used to create a burr hole at the following stereotaxic coordinates were used: 4.8 mm posterior to bregma, 1.7 mm lateral to the midline, and 8.2 mm ventral to the surface of the skull (Paxinos and Watson, 1986) (Figure 61) for injection into the substantia nigra region.
Figure 61: Diagram showing the three stereotaxic coordinates and the structures at 4.2mm posterior to bregma.

Briefly, the study design is outlined in Table. 11. Rats were either dosed 2ug of LPS or 4ug of dose per rat and cytokines stimulation was evaluated at 6 hour time point and 18 hour time point. The needle of a 5μl Hamilton syringe, containing the LPS solution, was then lowered -8.8 mm ventral to the surface of the skull and 1ul of LPS was injected using a motorized microinjection Harvard Apparatus Infusion Pump at a rate of 0.5ul/min. After the injection, the needle was kept in place for 2 min and then slowly pulled out to minimize efflux. LPS was prepared as a stock solution of 2 mg/ml in sterile PBS for 2 μg stock and 4mg/ml stock was used for higher dose. LPS stocks were stored in small aliquots at 4°C. Each rat received an injection of LPS dissolved in PBS into one side of the brain mostly right except for time course study
injection was performed on the left side. The contralateral side was left untreated and served as an internal control and was analyzed separately.

Table 12: Outline of study for establishing the LPS model of neuron-inflammation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Intranigral injection LPS- <strong>2ug per rat</strong></td>
<td>Study cytokines levels at <strong>6 hour time point</strong></td>
</tr>
<tr>
<td>2.</td>
<td>Intranigral injection LPS- <strong>2ug per rat</strong></td>
<td>Study cytokines levels at <strong>18 hour time point</strong></td>
</tr>
<tr>
<td>3.</td>
<td>Intranigral injection LPS- <strong>2ug per rat</strong></td>
<td>Study cytokines levels at <strong>6 hour time point</strong></td>
</tr>
<tr>
<td>4.</td>
<td>Intranigral injection LPS- <strong>4ug per rat</strong></td>
<td>Study cytokines levels at <strong>6 hour time point</strong></td>
</tr>
</tbody>
</table>

8.2.1.2 Cytokine profiling based on quantitative PCR (qPCR) assay for substantia nigra

After microdissection of substantia nigra from each rat brain, tissues was stored in RNAse/DNase free conical tubes and stored in -80°C refrigerator. Next day, the tissue samples obtained were homogenized along with 1mL of Qiazol™ (Invitrogen) in 12ml sterile homogenization tubes. After letting sample sit on ice for 5 minutes, 200 μL of chloroform was added to each tube and the tubes were vortexed for 30 seconds and then left on ice for another 10 minutes. Finally the tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C to separate the aqueous layer containing the mRNA from the organic layer containing the tissue proteins. The aqueous layer was carefully taken out of the tubes and added to another eppendorf tube containing equal volume of 70% (v/v) ethanol and gently mixed by swirling the tube up and down few times.
Total RNA was isolated and purified from brain tissue using RNA-Easy Lipid Tissue Mini Kit from Qiagen. The isolated mRNA from the tissue samples described above was quantified by UV spectrophotometry using the Nano-Drop Instrument (Thermo Fisher Corp.). Briefly, the mRNA samples were diluted 1:100 with nuclease treated water and 2μL of this sample was placed on the pedestal of the instrument. Optical density of the samples was then determined at 260nm and 280nm for RNA concentration. The absorbance ratio of 260/280 was used to calculate the purity of the obtained RNA sample. A range of 1.9-2 was considered as a quality check mark.

Gene quantitation assay using TaqMan gene expression master mix and TaqMan gene expression assays from Applied Biosystems (Foster, CA) are performed in a two-step quantitative-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from RNA.

2. In the PCR step, PCR products are amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ).

The cDNA synthesis was carried out as per the guidelines provided in the TaqMan® Reverse Transcription kit (Invitrogen, Carlsbad, CA) using 1 ug of total RNA amount. In order to perform the DNA amplification, the tubes containing the cDNA were placed on ice-bath and were subsequently used for qPCR. Quantitative PCR procedure was carried out in the following manner: Five μL of cDNA sample was added to 96 well optical plates to which was added 12.5 μL of TaqMan® Gene Expression Master Mix (Invitrogen, CA) and 6.25 μL of PCR grade water and 1.25ul of primer set(s) (TaqMan Gene Expression Assay (20×)) was added. Plates were covered with optical adhesive cover. Plates were centrifuged for 2min to spin down the contents.
and to eliminate any air bubbles. β-actin was used as the internal control in the PCR experiments. The rat specific assay primer for TNF-α, IL-2, IL-6, iNOS, and β–actin were obtained from Invitrogen. The samples were heated at 50°C for 1 minute for UDG activation, 95°C for 10 minutes for enzyme activation and 50 PCR cycles of 95°C for 15 sec,60°C for 1min was performed for denaturing and annealing. Fluorescence generated due to reaction amplification of target gene was determined on Light cycler® 480 PCR detection system. Samples were run in a barcoded 96-well format compatible with Light cycler® 480 PCR instrument. The samples were run in triplicates and data was analyzed using comparative threshold cycle (Ct) method by calculating ddct values for each treatment and results were expressed as % relative expression compared to β-actin as endogenous control.

8.2.2 Evaluation and comparison of intranasal delivery of CSA as solution and as nanoemulsion formulations

Freshly prepared solutions and nanoemulsion formulations of CSA were used for the study. Rats were briefly anesthetized using ketamine and xylazine and following sedation equivalent amount of CSA as solution or nanoemulsion was delivered into the nasal cavity of rats over a period of 30min using a 20ul pipette. Pre-treatment was performed 3 hours before LPS injection. LPS injection was performed after briefly sedating animals with isoflurane. After LPS injection rats were returned back to cage and food and water was supplied. After 6 hours post-LPS injection, rats were sacrificed and brain was dissected. Microdissection was performed using the adult rat brain slicer matrix and 1-2mm of the slices were dissected. SN region from both injected side and contralateral side was collected and was frozen immediately. Tissues samples were processed further after homogenization to perform qPCR. Data analysis was
performed and results were reported as % change in target cytokines normalized to β-actin and to the untreated animals. The treatment plan is shown in Table 13.

Table 13: Study design for evaluating the treatment effect of CSA dosed I.N in the LPS model of neuron-inflammation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Dosing at time $T_0$</th>
<th>Central Injection at Time (h)</th>
<th>Collection at Time(h)</th>
<th>Biochemical Read Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - PBS n=3</td>
<td>PBS</td>
<td>PBS at 3hrpost dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha,IL-2,iNOS, IL-6 both sides of SN</td>
</tr>
<tr>
<td>Control-LPS n=3</td>
<td>PBS</td>
<td>LPS at 3hrpost dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha,IL-2,IL-6,IL.N.OS, IL-1B both sides of SN</td>
</tr>
<tr>
<td>S-CSA 5MPK n=5</td>
<td>S-CSA</td>
<td>LPS at 3hrpost dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha,IL-2,IL-6,IL.N.OS, IL-1B both sides of SN</td>
</tr>
<tr>
<td>NE-CSA 5MPK n=5</td>
<td>NE-CSA</td>
<td>LPS at 3hrpost dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha,IL-2,IL-6,IL.N.OS, IL-1B both sides of SN</td>
</tr>
</tbody>
</table>

8.2.3 Evaluation and comparison of intranasal delivery of TNF-alpha siRNA as solution versus nanoemulsion formulations

As previously mentioned genetic manipulation of TNF or TNF receptors in mouse models of disease have provided valuable insight into the biological roles of TNF in the CNS. Small interfering RNA mediated knockdown of proinflammatory cytokines at the messenger RNA (mRNA) level offers an alternative therapeutic strategy to overcome neuro-inflammation. Although siRNA’s has a therapeutic potential, they have a challenge to be delivered intracellularly due to their high molecular weight, enzymatic instability, in physiological fluids. We have designed a nanoemulsion system containing siRNA, where siRNA has been pre-complexed with DOTAP lipid. We evaluated the anti-inflammatory efficacy of TNF-α silencing siRNA in J774 macrophages first and have shown siRNA administered in nanoemulsion formulation was successfully being delivered to brain via intranasal route. In this study we
compared the knockdown effect of TNF-alpha in an LPS induced model of Neuro-inflammation in rats.

We compared the silencing effect of siRNA delivered intranasal in the form of saline solution versus siRNA delivered as nanoemulsion. The study design is outlined below in Table 14. Briefly, siRNA was dosed intranasal 16 hours before LPS injection. The time-point was selected based on the higher uptake observed at 24 hours after intranasal dosing. Rat brain tissue samples were harvested after LPS injection and SN region from injection site and the contralateral site was dissected. Tissues were processed as described previously for qPCR analysis to study the biochemical changes in the cytokine levels at RNA levels.

Table 14: Study design for evaluating the treatment effect of TNF-alpha siRNA dosed I.N in the LPS model of neuron-inflammation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Dosing time</th>
<th>Central Injection Time (hr.)</th>
<th>Collection Time (hr.)</th>
<th>Biochemical Read Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control - PBS n=3</td>
<td>PBS at 3hr post dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha, IL-2, IL-6, iNOS both sides of SN, Apo test</td>
<td></td>
</tr>
<tr>
<td>2 Control-LPS n=3</td>
<td>PBS at 3hr post dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha, IL-2, IL-6, iNOS both sides of SN, Apo test</td>
<td></td>
</tr>
<tr>
<td>3 STNF in Saline 1.5MPK n=3</td>
<td>LPS at 16hr post dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha, IL-2, IL-6, iNOS both sides of SN, Apo test</td>
<td></td>
</tr>
<tr>
<td>4 SNE-TNF 1.5MPK n=3</td>
<td>LPS at 16hr post dosing</td>
<td>6 hour post LPS</td>
<td>Cytokines- TNF-alpha, IL-2, IL-6, iNOS both sides of SN, Apo test</td>
<td></td>
</tr>
</tbody>
</table>

8.2.4 Evaluation and comparison of combination efficacy of CSA and TNF-alpha siRNA dosed intranasal as solution versus as nanoemulsion

As most of the neurodegenerative diseases have a cascade of events which are often complex, a therapy which could affect multiple functions might be beneficial for treatment or control of neurodegenerative diseases. With that in mind, we evaluated the treatment effect from
combination of CSA and TNF-alpha silencing siRNA. Cyclosporine could potentially act as a neuroprotective agent by various mechanisms outlined earlier and TNF-alpha siRNA could keep the up-regulated TNF-alpha levels under control for a longer duration due to its effect at transcription level. Therefore, we designed this study to understand the synergistic effect of both the molecules in the LPS induced model of neuro-inflammation. Animals were pretreated with TNF-alpha silencing siRNA as solution or as nanoemulsion (Treatment 1) followed by pretreatment with CSA either as solution or nanoemulsion (Treatment 2). LPS was injected as outlined above into the SN region of brain on the right side. The study design is shown below in Figure 62. After 6 hours post LPS intranigrial injection, brain was collected and SN region was microdissected from both the injection side and the contralateral side. Brain samples were homogenized and tissues were processed as described previously for qPCR analysis to study the changes in gene expression of various cytokine at RNA levels.

Figure 62: Experimental study design for the combination study. Dosing regimen was performed in the order as described.

8.2.5 Studying the effect of intranasal delivery of CSA and siRNA on neuronal degeneration in rat substantia nigral induced by intranigral injection of lipopolysaccharide
We attempted to assess the neuro-protective effect of CSA and/or TNF-Alpha siRNA formulations in LPS induced neuroinflammation rat model. We used Sprague dawley (200-220gm) rats and designed this study as detailed here. Four groups of animals were established for immunohistochemistry: (i) Naive animals which received no treatment and no LPS injection; (ii) the vehicle-injected group received a single intranigral injection of 2ul of phosphate-buffered saline (PBS); (iii) the LPS-injected group received pretreatment with PBS and a single intranigral injection of LPS dissolved in vehicle after 3 hours of pretreatment; (iv) NE-CSA group received pretreatment with CSA nanoemulsion at 5mg/kg dose and LPS was injected after 3 hour post-treatment; (v) S-CSA solution-LPS-treated group received CSA solution followed by a single intranigral injection of LPS (vi) STNF group received siRNA in saline(16hours pre-LPS injection) at 1mg/kg dose (vii) NE-CSA/SNE group received TNF-Alpha siRNA nanoemulsion(16hours pre-LPS injection) at 1mg/kg dose and CSA nanoemulsion (3hours pre-LPS injection) followed by a single intranigral injection of LPS(2 ug) dissolved in vehicle at day 2. All animals were finally sacrificed 3 days post LPS intranigrial injection, whole brains were extracted and stored in 4% formalin until further processing for immunohistochemistry(IHC).

For intranigrial LPS injection, rats were anaesthetized with isoflurane and positioned in a small-animal stereotaxic apparatus to confirm position with brain atlas. For injection of LPS into the SN pars compacta, the following coordinates were used: 4.8 mm posterior to bregma, 1.7 mm lateral to the midline, and 8.2 mm ventral to the surface of the skull based on Paxinos and Watson rat brain atlas. LPS was prepared as a stock solution at 2 mg/ml in sterile PBS and stored in small aliquots at 4°C. Each rat received a 2ul injection of LPS dissolved in PBS onto one side of the brain and the contralateral side was used as control. The injection was conducted over a
period of 2 min and controlled by a motorized microinjection pump. After the injection, the needle was kept in place for 2 min.

Immunohistochemical staining was performed by the histopathology laboratory at Tufts Cummings School of Veterinary Medicine in Grafton, MA. Briefly, tissue samples were harvested and immediately fixed in 10% neutral buffered formalin. Tissues were processed and paraffin embedded. Slides were initially deparaffinized and rehydrated through graded alcohols to water. Brain sections were then incubated with 3% hydrogen peroxide (20 min) to block endogenous peroxidases. Antigen retrieval was performed on slides by first heating in Cell Marque Declere (citrate based) at steam setting for 15 mins. The slides are then let cool for 20 mins and rinsed in distilled water. After antigen retrieval, all slides were processed using a computer-controlled, automated stainer. Brain sections were sequentially incubated with the following reagents; biogenex protein blocker was used as blocking solution (20 min), primary antibody diluted in blocking solution (60 min). The primary antibodies used include a rabbit polyclonal antityrosine hydroxylase (anti-TH; Millipore (AB152) 1:200) for staining the dopaminergic neurons, antiglial fibrillary acidic protein (anti-GFAP; rabbit monoclonal Cell Marque (EP672Y) predilute for astrocytes recognition and ionizing calcium-binding adaptor molecule 1 (Anti-Iba1; rabbit polyclonal WAKO 019-19741 1ug/ml) for recognition of activated microglia. Sections were incubated with the primary antibodies for 1 h. The Cell Marque Polymer Detection System was used. Antigen amplified for 10 minutes and rinsed with PBS. The Cell Marque DAB chromagen was applied and incubated for 8 minutes and then rinsed three times with PBS. The slides were transferred to tap water and counterstained in hematoxylin.
Finally, the slides are then dehydrated through graded alcohols placed in xylene and coverslipped.

Slides were scanned at 40x on an iCys instrument (CompuCyte Corp, Cambridge, MA), a multilaser scanning cytometer. Images were first captured using a low resolution scan of the tissues to select an injection site using anti-GFAP staining. High resolution images were captured after selecting similar region of interest area on both sides of the brain within Substantia Nigra region. The fluorophore content (red: DAB staining for respective cell measured at 633nm, blue: nuclei acid stain for 488nm) was quantified and sorted in scatter plots. For quantitation presentation data was plotted as average intensity percent compared to contralateral site for each of the animals.

8.2.6 Statistical data analysis

Statistical significance of results was determined using student test with a 95% confidence interval (**p<0.05).

8.3 RESULTS AND DISCUSSION

8.3.1 Establishment of LPS induced rat neuro-inflammation model

Firstly, we wanted to establish the LPS neuro-inflammation model to use for further evaluating the effect of various intranasal delivered formulations. The concentration dependence of LPS-induced cytokines stimulation in the SN region was examined by injecting various amounts of LPS (2-4ug) into the rat SN. We measured the expression of mRNAs encoding for TNF-α and iNOS after LPS injection. Six hours after injection of 2ug of LPS, 1800% relative increase was observed in the TNF-alpha cytokine and more than 1600% relative increase in
iNOS levels were observed (Figure. 63A). Graphs shows the gene expression normalized to the β-actin of individual animals and further normalized to the PBS treated animals on non-injectable side. Injection of larger amounts of LPS resulted in a greater stimulation TNF-alpha and iNOS cytokines.

Further, the time dependence of the LPS-induced neurodegeneration in the SN was examined by injecting 2ug of LPS into the SN, and rats were sacrificed at different time points. As shown in Figure. 63B, no apparent higher stimulation of cytokines TNF-alpha and iNOS was observed at later time point of 18hours compared to 6 hour time point. Infact, the levels were found to be lower than the levels found at 6 hour time point. Based on these preliminary evaluations of inflammation markers, where we found that LPS-induced neuro-inflammation in rat SN was concentration- and time-dependent, we initiated the efficacy studies and used 2ug LPS dose and selected 6 hour time point for end point.
Figure 63: Quantitative PCR analysis of cytokines in SN region of brain post-LPS administration. LPS-induced stimulation of cytokines TNF-alpha and iNOS in the SN was concentration-(3A) and time-dependent (3B).

For the concentration dependence study (A), rats were injected with the indicated quantities of LPS, and their brains were harvested 6 hour later. For the time course study (B), rats were injected with 2ug of LPS, and their brains were removed at the indicated timed intervals (6 and 18hour).

Although, we used the rat contralateral SN site as an internal control to study the stimulation site specificity, we further assessed if the stimulation of inflammation markers is SN region specific or does the stimulation occurs in other regions of brain at 6 hour time point. We assessed the stimulation of cytokines in cortex region and compared to the SN region. Results showed that TNF-alpha cytokine reached highest stimulation in SN region (35000%) and comparatively lower levels were obtained in cortex (3000%). Upon evaluation of other cytokines, we found iNOS and IL-6 also showed highest stimulation in SN region compared to cortex region. Cytokine IL-1β and IL-2 showed lowest stimulation in the SN regions however the levels were higher in SN region compared to cortex region (Figure 64).
Figure 64: Quantitative PCR analysis of brain tissue cytokine profile between the SN and cortex brain region of LPS-induced rats at 6 hour time point, post-LPS administration.

In the SN regions, there was significant up-regulation of pro-inflammatory cytokines TNF-α, IL-1β, iNOS and IL-6 while comparatively lower levels of cytokines stimulation was observed in the cortex regions (n=3).

8.3.2 Evaluation and comparison of intranasal delivery of CSA as solution and as nanoemulsion formulations

We utilized the LPS-induced model and performed qPCR analysis to evaluate the cytokines profile in SN regions upon treatment with control and nanoemulsion formulation for CSA. Both the injectable site SN and contralateral side SN were dissected from brain after 6 hour post-LPS injection. Samples were then processed for total RNA extraction, cDNA synthesis and subsequent PCR amplification. Figure 65 represents the % relative cytokine levels for different treatment groups after 3 hours of pre-treatment with CSA. As indicated in the figure, the levels of pro-inflammatory cytokines for the LPS-induced rats and no treatment were found to be significantly higher than the treated groups. For instance, the % relative expression of TNF-
alpha, iNOS, IL-6 after LPS stimulation was found to be 19,177 +/- 2693%, 1,876 +/- 725%, 2,300 +/- 576%. CSA delivered as solution formulation was found to be slightly effective in exerting therapeutic effect at this time point where TNF-alpha reached 14,223 +/- 7305%, with higher variability within the levels. The considerable lower effect of the CSA-solution was demonstrated in the in vitro studies conducted in LPS-stimulated macrophages. On the other hand, NE-CSA significantly lowered the levels of TNF-alpha(2,786 +/- 328%) compared to the LPS-induced group and also compared to the CSA solution group which emphasizes the importance of a nanomeulsion based delivery system. The levels of other cytokines IL-2 and IL-6 were also found to be lowered however, the results were not significant. IL-2 is another cytokine specifically down regulated by CSA and has been studied to be unregulated by T lymphocytes. Upon LPS stimulation, apart from microglia there is evidence that lymphocytes can also infiltrate the injection site (Figure 60). NE-CSA showed inhibition of the IL-2 gene expression which is known to be involved in the maintenance of regulatory T cells and the also involved in the differentiation and survival of T cells. The therapeutic effects observed on IL-2 cytokine were based on the inhibition of the endogenous levels. It was later found that LPS is not an effective stimulator of IL-2 levels and hence if the levels of IL-2 are considerably increased using other toxin we might be able to see a higher effect. NE-CSA treatment was found to be very effective in inhibiting the expression of cytokines including TNF-alpha and was found to be slightly effective in down regulating other cytokines like IL-2, IL-6. There was no effect of NE-CSA or S-CSA on iNOS levels.
8.3.3 Evaluation and comparison of intranasal delivery of TNF-alpha siRNA delivered as solution versus as nanoemulsion

We evaluated knockdown effect of TNF-alpha siRNA upon intranasal delivery in LPS-induced model of neuro-inflammation. TNF-alpha siRNA when delivered in solution form (STNF) showed no inhibition when compared to TNF-alpha siRNA delivered as nanomeulsion (SNE). SNE reduced levels of TNF-alpha from approximately 39,600 +/- 6,900% relative expression to 26,500 +/- 1,640% after 16 hours of pretreatment with siRNA (Figure 66). The time
frame for pretreatment was defined based on the uptake of siRNA in brain during 6 hour time point to 24 hour time point. No effect observed on TNF-alpha knockdown of the saline solution could be attributed to the limited siRNA exposure in brain by saline solution.

Further, we also studied the effect of TNF-alpha knockdown using TNF-alpha siRNA on other cytokines due to the crosstalk between these different cytokines. Intranasal dosing of TNF-siRNA in saline solution didn’t show any effect on the other cytokines, however SNE showed inhibition of iNOS levels which were found to be significantly different compared to the no treatment group. SNE also showed down regulation of IL-6 cytokine, although the results didn’t reach statistical significance. As evident, siRNA nanoemulsion was able to provide inhibitory effect in SN region upon intranasal delivery and furthermore, it was interesting to observe the cross talk between TNF-alpha and other cytokine down regulation.
Figure 66: Inflammatory marker TNF-alpha, iNOS and IL-6 specific mRNA results showing transcript expression in the SN region of rat brain.

Graph comparing the pre-treatment with TNF-alpha siRNA in solution (STNF) and siRNA-loaded nanoemulsion (SNE). Data was plotted by considering the expression of control saline group as 100%. The values reported are mean ± SEM (n = 3).

8.3.4 Evaluation and comparison of combination efficacy of CSA and TNF-alpha siRNA dosed intranasal as solution versus as nanoemulsion

To understand if there is any synergistic effect of CSA and TNF-alpha siRNA treatment on neuroinflammatory cytokines, we evaluated the combination dosing effect of CSA and TNF-siRNA in rats. Pretreatment was performed first with siRNA followed by pre-treatment with
CSA where siRNA was dosed at 1.5mg/kg and CSA was dosed at 5mg/kg either as solution or nanoemulsion. Animals were injected LPS and brains were collected and processed for qPCR. Based on the PCR results in Figure 67, we observed inhibitory effect on TNF-alpha, iNOS and IL-2 cytokines and there was no effect on IL-6. TNF-alpha decreased from 30,000% expression levels to approximately 12,000% after the combination treatment with NE-CSA and SNE. This effect was found to be statistically significant compared to solution group effect and also to the non-treated group. It was interesting to obtain a synergistic effect from solution group where CSA and siRNA were dosed as solution. The levels of TNF-alpha were reduced from 30,000% expression to 21,700% expression levels which were statistically significant compared to the untreated group. Further, the synergistic effect on iNOS cytokine was also observed, levels reduced from 1,362% to 454% after pre-treatment with a combination of NE-CSA and SNE. Solution group also reduced iNOS levels from 1,362% down to 1,039%. Although reduction in iNOS level was reported, results didn’t reach significance due to low number of animals used and high variability observed for this particular cytokine. The levels for IL-2 cytokine were also found to be reduced from 101% to 78% after pre-treatment with NE-CSA and SNE, on the contrary levels of IL-2 seems to increase from 103% to 165% in case of solution group. Results were not found to be statistically significant and the increase in levels was within the standard error of the control untreated groups. Surprisingly, the levels for IL-6 didn’t vary upon nanoemulsion treatment or solutions treatment, even though we did observe some positive down regulation of the cytokines with the pretreatment with SNE and SNTF group. Individual dosing results although were found to be lowered compared to untreated group, there is less significance that could be given to the outcome due to high variability in those groups.
Figure 67: Inflammatory marker TNF-alpha, iNOS, IL-2 and IL-6 specific mRNA results showing transcript expression in the SN region of rat brain comparing combination effect of pretreatment with TNF-alpha siRNA in nanoemulsion (SNE) combined with CSA-loaded nanoemulsion.

Data was plotted by considering the expression of control saline group as 100%. The values reported are mean ± SEM (n = 3).

8.2.5 Studying the effect of intranasal delivery of CSA and siRNA on neuronal degeneration in rat substantia nigral induced by intranigral injection of lipopolysaccharide

LPS (2.0μg in 1μl of PBS) was stereotaxically injected into the rat SN to examine its effect on neurodegeneration. Whole brains were removed from these animals and coronal sections were taken through the nigral complex. The sections were double immunostained with an antibody against a neuronal nuclear protein to detect neurons in general and antibodies against TH to specifically detect dopaminergic neurons; GFAP for detection of astrocytes and IBAF1 for
microglia response. As shown in Figure 70B and 70D, immunostaining of a coronal section of the rat brain was significantly reduced compared to the contralateral site. On activation with LPS injection, microglia took up an amoeboid morphology which signifies activation of macrophages.

Figure 68: Immunohistochemical analysis of the naïve animals showing the integrity of TH-immunoreactive neurons (A and B) and microglia (C and D) in the rat SN.

In naïve animals TH immunoreactivity was found to be similar on both sides (Figure 68B) and no microglia activation was observed (Figure 68D). Animals injected with PBS showed no
significant damage to the TH-immunoreactive neurons in the SN. There was some microglia infiltration that was observed in PBS injected animals (Figure 69).

Figure 69: Immunohistochemical analysis of the effect of intranigrally injected PBS on the integrity of TH-immunoreactive neurons (A and B) and microglia (C and D) in the rat SN.
Figure 70: Immunohistochemical analysis of the effect of intranigrally injected LPS on the integrity of TH-immunoreactive neurons (A and B) and microglia (C and D) in the rat SN.

The injection of LPS caused significant damage to the fibers and a substantial loss of TH-immunoreactive neurons in the SN (A: Low magnification, B: High magnification). IBA1-positive cell response in control noninjected SN was typical resident microglia with two or three fine processes. LPS injected site showed activated microglia morphology with shorter and thicker processes than resident cells.
Pretreatment of CSA nanoemulsion showed positive effect against neurodenegation due to LPS (Figure 71B). Rats were dosed with NE-CSA intranasally 3 hours before LPS microinjection into SN region. NE-CSA showed higher number of TH-positive cells as compared to the control group with no treatment. Further, less number of the IBAF1-positive microglia infiltrated in the treated rats compared to non-treated rat (Figure 71D).

Figure 71: Effect of CSA nanomulsion (NE-CSA) on the LPS induced loss of TH-immunoreactive neurons and microglia in the SN region.
Treatment with S-CSA showed no major effect on the neuronal damage, although the levels of microglia activation was found almost similar to the NE-CSA treated animals (Figure 72B and 72D). When rats were pretreated with both CSA and TNF-Alpha siRNA nanoemulsion, SN regions showed improved effect against neuronal damage when compared to contralateral site (Figure 73B and 73D).

Figure 72: Effect of CSA solution (S-CSA) on the LPS induced loss of TH-immunoreactive neurons and microglia in the SN region.
Figure 73: Effect of TNF-Alpha siRNA (STNF) on the LPS induced loss of TH-immunoreactive neurons and microglia in the SN region.
Figure 74: Effect of CSA nanoemulsion (NE-CSA) and TNF-Alpha siRNA (SNE) on the LPS induced loss of TH-immunoreactive neurons and microglia in the SN region.
Based on the quantitation data comparing the contralateral site to the injection site in each animal, NE-CSA pretreatment showed positive effect against neuronal damage (Figure 73). Microglia activation was found to be reduced in the NE-CSA treated animals and also in the animals treated with both siRNA and CSA nanoemulsion (Figure 74).

Figure 75: Comparison of immunohistochemical data based on the integrity of TH-immunoreactive neurons in the rat SN region.

Figure 76: Comparison of immunohistochemical data based on the intensity levels of the immunoreactive microglia in the rat SN region. Data was calculated based on the intensity levels on the injection site and normalized to the contralateral site.
8.4 CONCLUSIONS

We have extended our in vitro experiments to animal studies and report herein that intranigral injections of LPS into rat result in a rapid stimulation of various inflammatory marker cytokines. Our therapeutic efficacy studies conducted in LPS-induced model of Neuroinflammation showed intranasal delivery of nanoemulsion incorporating therapeutic molecules like cyclosporine and TNF-alpha siRNA are capable of inhibiting stimulated markers of inflammation to greater extent over the other solution formulations treatments. Further, preliminary immunohistochemistry showed some prevention of inflammation-related nigrostriatal neurodegeneration using Cyclosporine nanoemulsion. Based on the combination studies, it was evident that some synergistic effect could be possible when downregulation of different inflammatory markers and protection against neuronal damage is needed.
CHAPTER 9

PRELIMINARY SAFETY EVALUATION OF CYCLOPSORINE AND TNF-ALPHA SILencing SIRNA CONTAINING NANOEMULSION FORMULATIONS UPON INTRANASAL ADMINISTRATION IN RATS

9.1 INTRODUCTION

In addition to evaluating delivery and therapeutic efficacy, it is of utmost importance to monitor the safety and tolerability of formulations delivered through the intranasal route. Although we have utilized omega3 fatty acid based lipids which are known to be biodegradable, the toxicity of the particulate system with other excipients still needs to be determined. To examine the safety, following study was designed for each treatment groups as solution and as nanoemulsion with controls: body weight monitoring, histopathological evaluation of nasal mucosa and histophathological evaluation of liver sections. It is important that intranasal delivery of particulate system does not have any toxic effect on the sensitive nasal mucosa. Although the levels of the drugs reaching any peripheral organs was found to be much lower via intranasal route compared to Intravenous route, we evaluated safety of liver organ as it is possible to get formulation passing though the esophagus into the GI tract and distribute to liver.

9.2 MATERIALS AND METHODS

9.2.1 Body weight changes upon administration of aqueous solution and nanoemulsion formulations

Periodic measurements of the body weight were performed upon injecting the control, solution and nanoemulsion based formulation containing siRNA or CSA via intranasal route of
administration on day 0 to day 3. Frequent body weight measurements were made through the course of the study. A total of two animals were used. The results were plotted as percent change in body weight as a function of day’s pre-treatment administration for all the treatment groups.

**9.2.2. Nasal tissue histopathological analysis**

To evaluate the toxic effect on the nasal mucosa, rats were first dosed with different test articles and after exsanguination at 6 hour time point post administration, the head was removed from the carcasses. The tissues samples were preserved in formalin fixative until histopathological processing. After fixation and decalcification, four tissue slices were taken at the following levels (Figure 68). 1) Immediately posterior to the upper incisor teeth 2). At the incisive papilla or the anterior nasal cavity 3). Premolar or middle part of the nasal cavity 4). At the middle of the first molar teeth or posterior part of the nasal cavity. The nasal tissues were processed in a conventional manner. Paraffin embedded tissues were cut into 5 μm sections and mounted on a glass slides. Tissue sections were dried and de-paraffinized using xylene substitute followed by decreasing concentrations of ethanol down to purified water. Sections were incubated in hematoxylin, rinsed with water, and incubated with 1% acid alcohol (clearing reagent). Sections were rinsed and incubated with 4% ammonia solution (bluing reagent). Sections were then incubated with Eosin followed by dehydration by two changes each in 95% ethanol and 100% ethanol followed by final change of xylene substitute. Tissues were cover slipped and digital image was captured using a light microscopy (n=2/treatment). Blinded analysis of toxicological profile and tissue damage, if any, was carried out by Dr. Jerry Lyon, a certified veterinary pathologist, at the Tufts University Veterinary School in Grafton, MA.
Figure 77: Diagrammatic representation of the dissected nasal cavity of rats exposing surface epithelium.

*Vertical lines through the figures represent the levels of the anterior surfaced of the frontal blocks that were examined for each specimen [70].*

### 9.2.3 Liver tissue histopathological analysis

Liver tissues samples were collected for histopathological analysis from rats after 3 day of post treatment of control PBS, CSA solution, siRNA solution and Nanoemulsion for CSA and siRNA. These tissue samples were preserved in formalin before analysis. Paraffin embedded tissues were cut into 5 μm sections and mounted on a glass slides. Tissue sections were dried and de-paraffinized using xylene substitute followed by decreasing concentrations of ethanol to down to purified water. Sections were incubated in hematoxylin, rinsed with water, and incubated with 1% acid alcohol (clearing reagent). Sections were rinsed and incubated with 4% ammonia
solution (bluing reagent). Sections were then incubated with Eosin followed by dehydration by two changes each in 95% ethanol and 100% ethanol followed by final change of xylene substitute. Tissues were cover slipped and digital image was captured using a light microscopy (n=1/treatment). Blinded analysis of toxicological profile and tissue damage, if any, was carried out by Dr. Jerry Lyon, a certified veterinary pathologist, at the Tufts University Veterinary School in Grafton, MA.

9.3 RESULTS

9.3.1 Body weight changes from solution or nanoemulsion formulation dosing

For the four groups: saline treated (control LPS) with LPS microinjection, CSA solution (S-CSA) or TNF-alpha siRNA solution (STNF) and combination treatment with CSA and siRNA nanoemulsion (SNE/NE-CSA) only slight difference in body weight was seen at day 1 and all of these groups started to gain weight after 1 day except for S-CSA group. Rats in the combination group showed a steady gain in weight after day 1 (Figure 69). These results suggest that the nanoemulsion formulation were all well tolerated in rats.
Figure 78: Body weight measurements to determine safety/tolerability profile upon single intranasal administration of the control and cyclosporine and siRNA nanoemulsion.

The values are reported as percent change in body weight as a function of pre-treatment weight of rats.

9.3.2. Nasal tissue histopathological analysis

The frequency of sneezing or discomfort due to dosing of the formulation, which is a sign of irritancy was recorded. Nanoemulsion formulations containing either CSA or TNF-alpha siRNA were found to be well tolerated with no signs of irritation or sneezing observed. In some cases, groups which received control PBS solution reacted more than the nanoemulsion formulation or solution formulations however the reaction frequency decreased upon multiple dosing. Based on the histopathological report all the normal structures of the nasal cavities were identified and assessed and no significant pathology is identified in any of the sections for any of the animals. Upon a closer view of the respiratory and the olfactory mucosal lining the nasal cavity, there were no significant changes found in both the epithelium lining the nasal cavity. The histopathologic findings were found to be consistent with the nasal cavities of all the rats being within normal limits (Figure 70).
Control (PBS only): Rat 1 Respiratory ciliated epithelium (arrow), Goblet cells (GC), Bone (B), Blood Vessel (BV).

Control (PBS only): Rat 1 Olfactory epithelium (arrows), Olfactory cells (OC), Supporting cells (SC), Basal cells (BC), Nerve Processes (N).

Rat 4: NE-CSA Respiratory Epithelium with goblet cells (arrow), Blood Vessel (BV)

Rat 4: NE-CSA Olfactory Epithelium (arrow), Bone (B), Nerves (N).

Rat 10: S-CSA Respiratory Epithelium (arrows)

Rat 10: S-CSA Olfactory Epithelium (arrow), Nerves (N), Bone (B)
Figure 79: Histology of Nasal respiratory and olfactory epithelium

1: R1 – Control: PBS treatment
2: R4 – NE-CSA: CSA nanoemulsion treatment
3: R10 - S-CSA: CSA solution treatment
4: R22 – STNF: TNF-siRNA delivered as saline solution
5: R28 – SNE: TNF-alpha siRNA pretreatment for 6 hour

There were no signs of epithelium toxicity. The histological features observed apparently fall within a normal variation.
9.3.3 Liver tissue histopathological analysis

As shown in **Figure 71**, there was moderate periacinar and diffuse hepatocellular vacuolation. Occasional multifocal aggregates of cells were present in portal areas consistent with extramedullary hematopoiesis (EMH). Mild extramedullary hematopoiesis and lipid vacuolation are considered as common incidental findings in the liver and are not thought to be related to the treatment as they were also observed in control naïve animals (R22). Hence, the liver tissues histopathological findings for treated groups are consistent with what is regarded as within normal limits.
Figure 80: Histology of liver tissues

1: R24 – PBS control Group, Normal hepatic architecture with central vein
2: R22 – S-TNF pretreatment 3 day: Normal hepatic architecture
3: R25 - NE-CSA pretreatment 3 day: Normal hepatic architecture
4: R27 – SNE, siRNA-TNF Nanoemulsion pretreatment 3 day: Liver with normal architecture, portal area
5. R26 – S-CSA, CSA solution pretreatment 3 day

9.4 CONCLUSIONS

In general, animals lost some amount of the body weight immediately after surgery and they started gaining weights from day 1 onwards. No significant loss in body weight was evident for any treatment groups compared to no treatment groups. Tissue histology did not show any abnormal findings in nasal cavity or in liver in any of the treatment groups. Based on this data, it was concluded that nanoemulsion formulation were well tolerated by the animals and were considered to be safe upon intranasal administration.
CONCLUDING REMARKS

Currently, drug delivery to brain has been facing various challenges and to counter acts those various alternatives have been investigated. Intranasal administration is an effective and non-invasive method of transport for biologic drugs; however, nasal route has various challenges which lead to limited absorption of macromolecules.

Therefore, in this study, our goal was to design, optimize, and characterize a non-viral delivery system which can come over the challenges of limited absorption in nasal mucosa and can also provide sustained down regulation of various cytokines overexpressed in neuro-inflammation. Initial formulation design and optimization experiments showed that omega-3 fatty acid lipid based nanoemulsion were capable of encapsulating higher quantity of drugs and also were able to provide enhanced cellular transport in nasal squamous cells. Both cyclosporine and TNF-alpha siRNA showed higher uptake in brain regions compared to distribution into blood. This offers a major advantage as systemic exposure of these drugs could lead to unwanted side effects. Furthermore, both cyclosporine and siRNA showed down regulation of cytokines when tested in LPS induced model of neuro-inflammation.

Overall, we have shown feasibility of using nanoemulsion as safe and effective drug delivery strategy to enhance delivery of biologics like cyclosporine and TNF-alpha siRNA through the nasal route to target brain. These findings open up an opportunity to target variety of neurodegenerative diseases underpinned by inflammation within the nervous system, where exposure of drugs is a major limitation.
CHAPTER 10

REFERENCES

33. Moore, G.E. and A.A. Sandberg, *Studies of a human tumor cell line with a diploid karyotype*.