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I, Mansi Krishan , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Toxicology (Environmental Health).

It is entitled:

Enhanced Intranasal Delivery of Gemcitabine to the Central Nervous System

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Enhanced Intranasal Delivery of Gemcitabine to the Central Nervous System

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ABSTRACT

Drug transport to the brain for the treatment of neurological diseases is a challenge due to impenetrable nature of the blood brain barrier (BBB). Intranasal (IN) drug administration is a non-invasive approach for rapid direct drug delivery from the nose to the central nervous system (CNS), thereby minimizing systemic exposure. The current study focuses on a strategy to enhance the delivery of the nucleoside drug gemcitabine (GEM) to the CNS via IN administration. Our approach took advantage of the fact that the BBB and olfactory epithelial tight junctions (TJs) share many proteins in common. We hypothesized that by transiently increasing the permeability of nasal epithelial tight junctions using the BBB permeabilizer papaverine (PV), we would increase the concentration of GEM reaching the brain extracellular fluid (BECF) following IN delivery, with the goal of delivering therapeutic concentrations of nucleoside drugs to the CNS. Experimental methods included IN administration of fluorescein isothiocyanate-dextran beads (FD4), *in-vitro* GEM recovery, *in-vivo* brain microdialysis for BECF collection, HPLC analysis to measure GEM in BECF, histopathology, western blot analysis and immunofluorescence localization. Distribution studies with FD4 showed significant deposition in the ethmoid turbinates, suggesting drug uptake through olfactory epithelium. Clinically relevant doses of PV (up to 1.4% IN) did not cause histological evidence of cytotoxicity or inflammation in nasal epithelia, lung, liver, spleen, or kidney. Pharmacokinetics of GEM in BECF for PV (1.4%) + GEM (50mg/kg) treated animals showed almost four fold increase in area under the curve as compared to no PV treatment group. Western blot analysis

suggested that IN PV treatment increased permeability through olfactory epithelial TJs by transiently decreasing the levels of TJ protein phospho-occludin. Immunofluorescence staining showed reversible alteration of occludin localization in olfactory epithelium following IN PV treatment. Thus, transient permeabilization of nasal epithelial TJs provides a non-invasive means to enhance delivery of nucleoside drugs to the CNS.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ANOVA	Analysis of variance
B	Blank
BBB	Blood brain barrier
BCSFB	Blood– cerebrospinal fluid barrier
bDNA	Branched DNA
BECF	Brain extracellular fluid
BTB	Blood tumor barrier
cAMP	Cyclic adenosine monophosphate
CE	Cerebellum
cGMP	Cyclic guanosine monophosphate
CLDN5	Claudin-5
C _{max}	Maximum or peak concentration
CNS	Central nervous system
CSF	Cerebrospinal fluid
ET	Ethmoid turbinates
FB	Forebrain
FD4	Fluorescein isothiocyanate-dextran
GEM	Gemcitabine
H&E	Hematoxylin and eosin
HB	Hindbrain
IN	Intranasal

ISF	Interstitial fluid
L	Ladder
MT	Maxilloturbinates
NB	Nerve bundles
NS	Nasal septum
NT	Nasoturbinates
OB	Olfactory bulb
OE	Olfactory epithelium
OECs	Olfactory ensheathing cells
OMP	Olfactory marker protein
ORNs	Olfactory receptor neurons
PDEs	Phosphodiesterases
PV	Papaverine
RE	Respiratory epithelium
S	Striatum
TJ	Tight junction
T _{max}	Time at which peak concentration is observed
ZO-1	Zona occludens 1

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CHAPTER 1

INTRODUCTION

Delivery of therapeutics to the brain to treat neurological diseases and other CNS-related disorders is a challenge due to the impenetrable nature of the BBB. According to the Central Brain Tumor Registry of the United States (1985-1994), the prevalence of malignant brain tumors was 29.5 per 100,000 (F. G. Davis et al. 2001). In third-world countries, including those in sub-Saharan Africa, where access to sophisticated, continuous medical care is rare, the incidence of dementia in AIDS patients was estimated to range from 31-62% of those infected (Wong et al. 2007; Schifitto et al. 2007). Many CNS diseases, including stroke, Alzheimer's disease, HIV, multiple sclerosis, vasogenic edema, viral infection and brain tumors have been found to be associated with compromised BBB (Krizanac-Bengez, Mayberg, and Janigro 2004; Sköld et al. 2005). Nucleoside drugs are important tools in treating many important diseases, including HIV/AIDS and various cancers. While oral or injection treatment with these drugs make them systemically bioavailable, they do not penetrate efficiently from the bloodstream into the brain due to the BBB. A good understanding of regulation of the BBB is required for effective delivery of drugs to the CNS.

Blood brain barrier and its barrier functions

The BBB comprises of endothelial cells that form the walls of the capillaries. The total area of blood-brain exchange for the average human adult is between 12 and 18 m² (Bridges 2007). It prevents the net movement of polar and high

molecular weight molecules from the bloodstream into the brain using a combination of efflux transporters and TJ proteins on endothelial cells (Ge, Song, and Pachter 2005; Hawkins and Davis 2005; Hawkins and Egleton 2006; Persidsky et al. 2006). The proteins involved in forming TJs include claudins, occludin, junctional adhesion molecules, and cytoplasmic proteins such as zona occludens (Persidsky et al. 2006; Ueno 2007; Furuse et al. 1993; Wolburg and Lippoldt 2002; Stamatovic, Keep, and Andjelkovic 2008). There is another interface in the brain called the blood– cerebrospinal fluid barrier (BCSFB), is formed by the epithelial cells of the choroid plexus facing the cerebrospinal fluid (CSF). The epithelial cells of the choroid plexus secrete CSF into the brain ventricular system (Brown et al. 2004), while the secretion from the capillary endothelium of the BBB is responsible for remainder of the brain extracellular fluid, the interstitial fluid (ISF) (Cserr et al. 1981; Cserr and Patlak 1992; Abbott 2004; Dolman et al. 2005). The ISF and CSF are in equilibrium as they are free to communicate at several locations. Previous studies have estimated the contribution of ISF to CSF as 10–60% (Milhorat et al. 1971; Davson and Segal 1996).

The BBB and BCSFB are the two main barriers that maintain brain homeostasis by restricting the entry of potential toxins and drugs in the brain parenchyma. Some of the factors, which determine the entry and distribution of drug into the CNS, are transport across the barriers (BBB and BCSFB), physiochemical properties of the drug, drug metabolism, plasma protein binding, CSF turnover,

pathological conditions and presence of influx-efflux transporters (E. C. M. de Lange and Danhof 2002).

Drug delivery to the brain

Studies have shown that BBB blocks the delivery of more than 98% of CNS therapeutics (William M. Pardridge 2001; W. M. Pardridge et al. 1992). Different strategies have been used to deliver drugs to the brain. These include invasive methods such as intracerebro-ventricular infusion or intracerebral implants (Bobo et al. 1994; Kroll et al. 1996) and non-invasive methods such as disruption of the BBB using ionizing radiations, vasoactive drugs and by infusing hypertonic agents (Griffin, Rasey, and Bleyer 1977; Levin, Edwards, and Byrd 1979; Olesen and Crone 1986; Neuwelt and Rapoport 1984; Emerich et al. 1998). Neurosurgical resection of brain tumors often leads to recurrence of brain cancer and severely shortens the life expectancy (Rich and Bigner 2004). Intracerebral implants are effective against very small tumors and ineffective against larger tumors because they do not allow anti-cancer agents to diffuse and reach the small pockets of tumor cells well away from the tumor core (Fung et al. 1996). Other conventional treatment methods like intravenous chemotherapy and radiotherapy have most of the times proven unsuccessful majorly because the drugs cannot cross the BBB in significant amount (William M. Pardridge 2001). Several innovative methods for drug delivery have been developed including nanoparticles (Ferrari 2005), microparticles as carriers of anti cancer agents, encapsulating anti cancer drugs in liposomes, PEG technology and monoclonal

antibodies for delivery of anti tumor drugs (William M. Pardridge 2001). Another complex strategy is the use of vectors, which carry modified proteins, peptides or monoclonal antibodies that undergo receptor-mediated transcytosis and allow delivery of therapeutics to normal brain tissue (William M Pardridge 2003). It has been demonstrated that activation of potassium channels induces accelerated formation of transport vesicles in brain tumor capillary endothelium and tumor cells and is largely responsible for enhanced delivery of drugs across brain tumor capillaries to the tumor tissue (Hu et al. 2007; Ningaraj et al. 2002; Hashizume and Black 2002; Black and Ningaraj 2004). Presence of efflux transporters which belong to the family of ATP-binding cassette (ABC) transporters such as breast cancer resistance protein (Breedveld et al. 2005), RLIP76/RALBP1 (recently described non-ABC multi-specific transporters) (Awasthi et al. 2005), P-glycoprotein (P-gp), and multi-drug resistance-associated protein at the BBB are involved in active export of the drugs from the brain to the blood. It has been shown that P-gp plays a critical role in clinical resistance to chemotherapy in primary brain tumors (Régina et al. 2001).

In order to study drug transporter mediated uptake from nose to the brain, we conducted a study to localize and compare the levels of different transporters in rat nasal epithelium (Genter et al. 2010). We used branched DNA (bDNA) technology (bDNA) to compare the level of expression of nine transporters (ENT1 and ENT2; OCT1, 2, and 3; OCTN1; OATP3, and MDR1 and MDR4) in nasal respiratory mucosa, olfactory mucosa, and olfactory bulb to the level of

expression of these transporters in the liver and kidney (**Table 1**). Using immunohistochemistry, we immunolocalized the transporters with high expression in the nasal respiratory mucosa or olfactory. ENT1 and ENT2 expression was relatively high in nasal epithelia and olfactory bulb, which may explain the uptake of intranasally-administered nucleoside derivatives observed by other investigators (Genter et al. 2010; Farrell et al. 2009). OATP3 immunoreactivity was high in olfactory epithelium and olfactory nerve bundles, which suggests that OATP3 substrates may be candidates for intranasal administration. We did not identify mechanism to increase the expression of transporters in the nasal epithelia or upregulate the transporters genes, so we focused on another transport route, the paracellular route.

Transporter	Tissue	Expression relative to liver
ENT1	Olfactory epithelium	1.8
	Respiratory epithelium	1.7
	Olfactory bulb	2.3
ENT2	Olfactory epithelium	2.0
	Respiratory epithelium	2.8
	Olfactory bulb	7.0
OCT2	Olfactory epithelium	32.3
	Respiratory epithelium	9.8
	Olfactory bulb	0.2
MRP1	Olfactory epithelium	173.3
	Respiratory epithelium	100.0
	Olfactory bulb	333.3
MRP4	Olfactory epithelium	67.6
	Respiratory epithelium	56.2
	Olfactory bulb	12.6
OATP3	Olfactory epithelium	46.3
	Respiratory epithelium	42.2
	Olfactory bulb	2.1

Table 1: Transporter expression in rat nasal epithelia using bDNA amplification

Intracarotid infusion of hyperosmotic agents is one means to increase BBB permeability, and this method was found to be associated with downregulation of TJ proteins present at the BBB (William M. Pardridge 2001; Wolburg and Lippoldt 2002; Argaw et al. 2009). Increased permeability of the entire BBB, while potentially beneficial for drug delivery purposes, can have adverse effects, including the potential for brain edema and the passage of infectious agents from the bloodstream into the brain. Thus, we used a more localized approach, namely perturbing only the nasal epithelial TJs, to enhance drug delivery to the CNS.

Intranasal route

The oral route is considered to be the most desirable route for drug administration for systemic drug delivery. But poor stability of pharmacologically active compounds in gastrointestinal fluids, poor intestinal absorption and/or extensive hepatic first-pass elimination, low bioavailability and the inability to cross the BBB, prompted the search of more effective routes (Dressman, Thelen, and Jantravid 2008; Illum 2003). The IN route is becoming more popular for administration of pharmaceutical agents and vaccines. The widespread interest in IN route arises from the particular anatomical, physiological and histological characteristics of the nasal cavity. The highly vascularized large nasal mucosa provides potential for rapid drug absorption; quick onset of action and direct access to the CNS thereby avoiding the gastrointestinal and hepatic pre-systemic metabolism, enhancing drug bioavailability (Pires et al. 2009;

Leonard et al. 2007; Dahl and Mygind 1998). From the viewpoint of patients, the IN route offers several practical advantages such as non-invasiveness, essentially painless, ease of drug administration and greater acceptance, particularly by children (Rapoport and Winner 2006). Despite its advantages, the nasal route has some limitations too. The enzymatic degradation of drugs might occur in the nasal mucosa and the relatively low volume of the nasal cavity restricts the amount of drug formulations to about 100-150 μ l (in rodents, volume is considerably higher in primates) and can be a problem in case of nasal delivery of high doses of poorly water-soluble drugs (Costantino et al. 2007). Recent advances in pharmaceutical technology have helped overcome these drawbacks by making use of enzymatic inhibitors, synthesis of prodrugs and/or the use of enhancers or bioadhesive polymers to increase drug permeability and residence time in nasal cavity. However, there is a possible risk to develop nasal irritation and/or systemic toxic effects due to the use of enzyme inhibitors and some nasal enhancers (Hosoya et al. 1994).

Intranasal drug delivery

Many successes have been made in IN drug delivery in the past few years. Drugs have been administered intranasally for local delivery, systemic delivery, as nasal vaccines and most importantly to reach the CNS. IN route is the most common choice for the treatment of topical nasal disorders such as antihistamines and corticosteroids for rhinosinusitis, and nasal decongestants for cold symptoms (Illum 2003; Costantino et al. 2007). Studies have shown that, for

some drugs, IN administration is a more effective way to systemically deliver drugs as opposed to oral and parenteral administration (Leonard et al. 2007; Varshosaz, Sadrai, and Heidari 2006; Ikechukwu Ugwoke et al. 2000). Examples of drugs administered as nasal formulations to achieve systemic effects include analgesics (morphine) (Illum 2003; Illum 2002; Stoker et al. 2008); cardiovascular drugs such as carvedilol (Patil and Sawant 2009); hormones such as levonorgestrel (W.-X. Ding et al. 2007), progesterone (Rathnam, Narayanan, and Ilavarasan 2008) and insulin (Varshosaz, Sadrai, and Heidari 2006; S. Yu et al. 2004; Jian Wang, Tabata, and Morimoto 2006); anti-inflammatory agents such as indomethacin (Karasulu et al. 2008; Onischuk et al. 2008) and ketorolac (Leykin et al. 2008; Moodie et al. 2008); and antiviral drugs (acyclovir) (Shao et al. 1994; Yang, Gao, and Mitra 2001; Alsarra, Hamed, and Alanazi 2008; Alsarra et al. 2009). The first site of contact with inhaled antigens is the nasal mucosa and thus, it is being use extensively for vaccination, especially against respiratory infections (Costantino et al. 2007). The systemic levels of specific immunoglobulin G and nasal secretory immunoglobulin A were enhanced after IN administration as compared to parenteral route (E. Karasulu et al. 2008; Huang et al. 2004; Sharma et al. 2009; Slütter, Hagenaars, and Jiskoot 2008). However, after nasal administration, secretory immunoglobulin A was detected in other mucosal secretions, which might prove to be useful in combatting virus transmitted through other mucosal sites, such as human immunodeficiency virus (Bråve et al. 2008) and hepatitis B virus (Borges et al. 2008).

Intranasal drug delivery to the brain

The nose also serves as a direct route to the brain. In 1937, W.F. Faber demonstrated for the first time the existence of the olfactory pathways by placing Prussian blue dye in the nasal cavity of rabbits and observed it later in both the olfactory nerve and the brain (Faber 1937). Decades later, Faber's experiments were performed in reverse- dyes injected into the brain ventricles of rabbits and monkeys showed that the CSF is drained into the lymphatic vessels and the nasal mucosa via the olfactory neurons (Jackson, Tigges, and Arnold 1979; Yoffey 1958). Thus, these results laid the foundation for present day studies for IN drug delivery to the brain.

The nasal cavity is divided into four main regions - the nasal vestibule, the atrium, the respiratory region, and the olfactory region (Illum 2003; Illum 2002). The respiratory epithelium covers most of the nasal mucosal area and is considered the most important section for delivering drugs systemically (Chien and Chang 1987). The olfactory mucosa is located posteriorly in the nose, between the superior turbinate and the roof of the nasal cavity, just underneath the cribriform plate of the ethmoid bone (Harkema, Carey, and Wagner 2006). The olfactory mucosa is found at the junction between the external environment and the systemic circulation and the brain tissue. It is rich in many unique forms of xenobiotic metabolizing cytochrome P-450 enzymes such as human CYP2A13, rat CYP2A3, mouse CYP2A5, rat and mouse CYP2G1 (X. Ding and Dahl 2003; X. Ding and Kaminsky 2003; Ling et al. 2004; Zhuo et al. 2009). In humans, the olfactory mucosa covers 3-10 % of the total nasal surface area

and is composed of three main cell types: olfactory receptor neurons (ORNs), supporting cells, and basal cells. ORNs, which function primarily as sensory cells, also provide a means for the direct transport of agents to the CNS (Dhuria, Hanson, and Frey 2010). The ORNs are bipolar neurons and their dendrites extend into the mucous layer of the olfactory epithelium. The axons of these neurons extend centrally through the lamina propria and through perforations in the cribriform plate of the ethmoid bone, which separates the nasal and cranial cavities and pass through the subarachnoid space containing CSF and terminate on mitral cells in the olfactory bulbs (Dhuria, Hanson, and Frey 2010). These neural projections form multiple synapses and extend into different brain regions including the olfactory tract, anterior olfactory nucleus, piriform cortex, amygdala, and hypothalamus (Dhuria, Hanson, and Frey 2010; Buck 2000). The ORNs have this unique ability to regenerate every 3-4 weeks as they are in direct contact with external environment and are exposed to toxins and infections. They regenerate from the basal cells in the olfactory epithelium and it has been suggested that ORNs regeneration is one of the defense mechanisms of the body to protect the CNS from viral infections (Mori et al. 2002; Mori et al. 2006; Brauchi et al. 2006).

Nasal transport routes

Therapeutics can reach the CNS via nasal route by two possible transport mechanisms i.e. transneuronal (intracellular) and/or paracellular (extracellular) pathways along the olfactory and trigeminal nerves that innervate the olfactory

epithelium (Frey II 2002; Thorne et al. 2004; Dhanda et al. 2005, 200; W. Pardridge 2005; Dhuria, Hanson, and Frey 2010). Olfactory epithelial TJs express many proteins also localized to the BBB, including zona occludens 1 (ZO-1), occludin, and claudin-5 (CLDN5) (Wolburg et al. 2008; Miragall et al. 1994; Hussar et al. 2002). The intracellular or transneuronal pathway is considered to be a slow route as it takes several hours to days for a drug to appear in the olfactory bulbs and other brain areas. In the transneuronal route, the drug uptake into ORNs occurs by passive diffusion, receptor-mediated endocytosis or adsorptive endocytosis, followed by slower axonal transport (Baker and Spencer 1986; Broadwell and Balin 1985; Kristensson and Olsson 1971). Studies have demonstrated the transport of small, lipophilic molecules such as gold particles and substances with receptors on ORNs by the transneuronal pathway in ORNs (De Lorenzo 2008; Thorne et al. 1995; Baker and Spencer 1986; Itaya, Van Hoesen, and Barnes 1986; M. T. Shipley 1985). Special Schwann cell-like cells called OECs envelope the axons of ORNs and create fluid-filled perineural channels that which is continuous with the subarachnoid space of the brain and filled with CSF (Li, Field, and Raisman 2005). The paracellular or extracellular pathway is significantly faster and the molecules pass through the paracellular spaces in between the olfactory epithelia cells into the perineural space, which is continuous with the subarachnoid space and in direct contact with the CSF (Frey II 2002; Balin et al. 1986).

Successes in intranasal delivery to brain

Many drugs have been successfully delivered to the CNS. Potent therapeutics those are active in nanomolar range work best with IN delivery (Dhanda et al. 2005). Some of the examples of the drugs which have been successfully delivered to the brain are neurotrophins NGF (Vaka et al. 2009) and insulin-like growth factor [IGF]-1 (Thorne et al. 2004); neuropeptides such as exendin (Banks, During, and Niehoff 2004); cytokines (interferon β -1b (Ross et al. 2004)) and erythropoietin (Y.-P. Yu et al. 2005); polynucleotides (DNA plasmids (Han et al. 2007)) and genes (Draghia et al. 1995); and small molecules (chemotherapeutics (D. Wang, Gao, and Yun 2006)) and carbamazepine (Barakat, Omar, and Ahmed 2006). A single IN insulin dose has shown to improve verbal memory in Alzheimer's patients with no change in blood levels of insulin or glucose (Banks 2012; Reger et al. 2006). IN neurotrophic factor and its active peptide fragment NAP are now in phase II clinical trials as a potential treatment for Alzheimer's disease and mild cognitive impairment (Matsuoka et al. 2007; Gozes et al. 2000).

Current study

Numerous investigators have investigated various strategies to enhance IN drug delivery to the CNS. Many of these strategies resulted in severe, and often irreversible, damage to the olfactory mucosa (S. S. Davis and Illum 2003). Therefore, we decided that we would attempt to enhance intranasally administered nucleoside drug delivery to the brain by transiently increasing

paracellular transport between cells of the olfactory mucosa. Olfactory epithelial TJs express many proteins also localized to the BBB, including zona occludens 1 (ZO-1), occludin, and claudin-5 (CLDN5) (Wolburg et al. 2008). Because of the similarity in structure between the BBB and olfactory epithelial TJs, we propose that a strategy that has been demonstrated to be effective in transiently increasing permeability of the BBB will also transiently perturb olfactory epithelial TJs, thereby enhancing the delivery of IN-administered drugs to the CNS.

Extensive literature searches were done to learn about the past and recent studies on enhanced IN drug delivery (**Table 2**). Most of the studies targeted systemic drug delivery and did not use paracellular route as a pathway. Since the aim of this study is to increase paracellular permeability through nasal epithelial TJs, we decided to use papaverine as a nasal permeabilizer.

Molecule/Drug treatment	Enhancer	Disease	Species	Ref.
Acyclovir	Hydroxypropyl b-cyclodextrin >sodium deoxycholate> sodium>caprate > sodium> tauroglycocholate >EDTA	Herpes simplex, varicella zoster, cytomegalovirus, and Epstein Barr virus infections	Rat	(Chavanpatil and Vavia 2004)
AT1002, a six- amino acid fragment of Zonula occludens toxin	Carrageenan as bioadhesive polymer and dextrose as stabilizer	Proof of concept	Rat	(Song and Eddington 2012)
Calcitonin	N- acetyl-L- cysteine	Proof of concept	Rat, rabbit, dog	(Matsuyama et al. 2006)
Cannabidiol	Polyethylene glycol 400	Chronic and breakthrough pain	Rat	(Paudel et al. 2010)
Erythropoietin, human growth hormone, and salmon	Lipoamino acids	Proof of concept	In vitro	(Bijani et al. 2012)

calcitonin				
Exendin-4	Polyethylene glycol	Type 2 diabetes	Mouse	(Kim et al. 2012)
Fentanyl	Pectin, chitosan and chitosan-poloxamer 188	Proof of concept	Human	(Fisher et al. 2010)
Fexofenadine	Chitosan-coated liposome	Allergic rhinitis	In vitro, rat	(Qiang et al. 2012)
Fluorescein isothiocyanate-labeled 4 kDa dextran (FD-4)	Sodium hyaluronate	Proof of concept	In vitro, rat	(Horvát et al. 2009)
Fluorescent isothiocyanate-labeled dextran	N-acetyl-l-cysteine + nonionic surfactant, polyoxyethylene lauryl ether (laureth-25)	Proof of concept	Rat	(Matsuyama et al. 2006a)
Galanin-like peptide (^{125}I -GALP)	Cyclodextrins	obesity and related conditions	Mouse	(Nonaka et al. 2008)
Gemcitabine	Polybutylcyanoacrylate nanoparticles	Anti-tumor	Rat	(C.-X. Wang et al. 2009)

	coated with polysorbate-80			
Gemcitabine	Papaverine	Proof of concept	Rat	(Krishan et al. 2013)
Granisetron	Hydroxypropyl-cyclodextrin and sodium carboxymethyl cellulose	Treatment of nausea and vomiting following chemotherapy	Primary human nasal epithelial cells in vitro	(Cho et al. 2010)
Growth differentiation factor 5	Lipid microemulsion of olive oil and phosphatidyl serine	Parkinson's disease	Rat	(Hanson et al. 2012)
Indomethacin	Polyvinylpyrrolidone, citric acid, sodium taurocholate	Anti-inflammatory effects	In vitro, ex vivo, rat	(H. Y. Karasulu et al. 2008)
Insulin	Oligoarginine-linked poly (N-vinylacetamide-co-acrylic acid)	Diabetes/obesity	Mouse	(Sakuma et al. 2010)
Insulin	Starch nanoparticles with	Streptozotocin-induced diabetic	Rat	(Jain et al. 2008)

	sodium glycocholate	rats		
Insulin	Aminated gelatin	Proof of concept	Rat	(Toshinobu Seki et al. 2005)
Insulin	PEG-grafted chitosan nanoparticles	Proof of concept	Rabbit	(X. Zhang et al. 2008)
Insulin	Chitosan-N- acetyl-L-cysteine nanoparticles	Proof of concept	Rat	(X. Wang et al. 2009)
Insulin	Polysaccharide nanoparticles consisting of chitosan and cyclodextrin (CD) derivatives- (sulfobutylether- β -CD or carboximethyl- β - CD)	Proof of concept	Rabbit, in vitro	(Teijeiro-Osorio, Remuñán- López, and Alonso 2009)
Isosorbide dinitrate	Poloxamer 188	Angina pectoris	Rat	(Na et al. 2010)
Lorazepam	Vinyl polymer- coated	Maximize speed of onset and	Rabbit	(Zhao et al. 2012)

	microparticles	minimize carry-over sedation		
Lysozyme	Hydroxypropyl-methylcellulose, hydroxypropyl-cyclodextrin, d-alpha-tocopheryl polyethylene glycol 1000 succinate	Delivery of therapeutic proteins	Human nasal epithelium in vitro	(Cho et al. 2011)
Midazolam	Cyclodextrins and chitosan	Proof of concept	Human	(Haschke et al., 2010)
Morphine	Nanoparticles consisting of a polysaccharide core surrounded by a lipid bilayer	Analgesia	Mice	(Betbeder et al. 2000)
Morphine, fentanyl	Pressurized olfactory delivery device	Analgesia	Rats	(Hoekman and Ho 2011)
Nelfinavir, mannitol	Pressurized olfactory delivery device	Proof of concept	Rat	(Hoekman and Ho 2011)
Nerve growth factor	Mutation at residue R100	Neurodegenerative diseases	Mouse	(Capsoni et al. 2012)

Olanzapine	Poly (lactic-c-glycolic acid) nanoparticles	CNS disorders	Ex vivo	(Seju, Kumar, and Sawant 2011)
Ondansetron HCl	Solid lipid nanoparticles	Management of chemotherapy induced postoperative nausea and vomiting	In vitro	(Joshi et al. 2012)
Peptide YY 3–36 (PYY3–36)	Novel tight junction modulating peptide (PN159)	Proof of concept	Rabbit	(Chen et al. 2006)
Pituitary adenylate cyclase activating polypeptide	Cyclodextrins	Alzheimer's disease	Mouse	(Nonaka et al. 2012)
Recombinant hirudin-2 (rHV2)	Chitosan	Anticoagulant, antithrombotic activities	Rat, in vitro	(Zhang et al. 2005)
Recombinant human growth hormone (rhGH)	<i>N</i> -trimethyl chitosan chloride and a patented fatty acid-based	Proof of concept	Rat	(Steyn, du Plessis, and Kotzé 2010)

	delivery system			
Recombinant iduronidase (IDUA; in the form of laronidase)	Adeno-associated virus vector encoding IDUA	Lysosomal storage diseases	Mouse	(Wolf et al. 2012)
Rivastigmine (RHT)	Chitosan nanoparticles	Alzheimer's disease	Rat, in vitro	(Fazil et al. 2012)
<i>Streptococcus equi</i> proteins	Poly (L-lactic acid) and glycol chitosan nanospheres	Strangles	Mouse	(Florindo et al. 2009)
Tacrine	Mucoadhesives(α lbumin nanoparticles carrying cyclodextrins	Alzheimer's disease	In vitro, proof of concept	(Luppi et al. 2011)
Thymopentin (TP5)	Chitosan and bacitracin	Modulator for immunodeficiencies	Rat	(Jing Wang et al. 2006)
Urocortin peptide	Odorranalectin	Parkinsonism	Rats	(Wen et al. 2011)

Table 2: Examples of strategies to increase delivery of intranasally- administered drugs.

Papaverine (PV) is a potent vasodilator acting as a phosphodiesterase inhibitor and is used in human medicine for indications ranging from treatment of cerebral vascular constrictions to treatment of erectile dysfunction (Kaku et al. 1992; Schuknecht et al. 1999; Mooradian et al. 1989; Fandino et al. 1998; Minami, Kuwamura, and Tamaki 2001). Phosphodiesterases (PDEs) are intracellular enzymes that regulate cyclic nucleotide signaling by phosphodiesterase-catalyzed cyclic nucleotide catabolism. These enzymes are involved in the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphates (cGMP) into their respective nucleotide monophosphates (Francis, Turko, and Corbin 2001). There are 11 known families of PDEs that are encoded by 21 different genes (Lugnier 2006). Of all the known PDEs, the current study relates to PDE10A, as PV is a specific inhibitor of PDE10A with an IC_{50} value of 36 nM (Siuciak et al. 2006). The characteristic properties of PDE10A are its dual specificity and unique localization in mammals relative to other PDE families. PDE10A can convert both cAMP to adenosine monophosphate and cGMP to guanosine monophosphate (Fujishige et al. 1999). Previous studies have demonstrated high expression of PDE10A in the brain, particularly in striatal medial spinal neurons, with variable expression in the testis, epididymal sperm and enteric ganglia (Fujishige et al. 1999; Fujishige, Kotera, and Omori 1999). In human brain, PDE10A is highly expressed in caudate nucleus and putamen of striatum; in other mammalian species, it is found in the striatal complex including caudate nucleus, nucleus accumbens and olfactory

tubercle (Z. Xie et al. 2006; Coskran et al. 2006; Seeger et al. 2003; Soderling, Bayuga, and Beavo 1999).

PV, which is a PDE10A inhibitor, has displayed anti-schizophrenic activity and anti-psychotic activity in different animal models of neurological disorders (Siuciak et al. 2006; Grauer et al. 2009). PV has also been shown to transiently down regulate BBB proteins in rats (A K Bhattacharjee et al. 2001; Platz et al. 2008; Z.-H. Wang, Xue, and Liu 2010). When administered via the external carotid artery, PV (0.2%) caused an increase in cerebrovascular permeability to ^{14}C -sucrose lasting ~3 h (A K Bhattacharjee et al. 2001). Similarly, intracarotid infusion of 0.15% PV caused increased blood-tumor-barrier (BTB) permeability lasting approximately 2 h, with downregulation of occludin, CLDN5, and F-actin (Z.-H. Wang, Xue, and Liu 2010). We hypothesized that IN-administered PV would also safely and transiently perturb nasal epithelial TJs.

Figure 1: Papaverine structure (*From PubChem Compound*)

In the present study we used *in vivo* microdialysis to investigate the potential for PV to enhance delivery of IN-administered gemcitabine, a model nucleoside chemotherapeutic drug to the CNS. Nucleoside analogs such as Gemcitabine and Sapacitabine have been used as potent radiosensitizers for the treatment of solid tumors (Sigmond et al. 2009; Anderson et al. 2008). Gemcitabine, 2'-deoxy-2', 2'-difluorocytidine (**Figure 2**), is a nucleoside anticancer drug which has shown to be effective in the treatment of some solid tumors (S. M. De Lange et al. 2004; Santoro et al. 2000). It has been shown that gemcitabine passes the blood-tumor barrier in glioblastoma multiforme patients and gemcitabine concentrations were high enough to enable radiosensitization (Sigmond et al. 2009). A previous study has demonstrated increased GEM uptake into brain tumors as compared to normal brain tissue, due to differential permeability of tumors (Sigmond et al. 2009; Apparaju, Gudelsky, and Desai 2008). We hypothesize that increasing the permeability of nasal epithelial TJs using PV will enhance the delivery of GEM from the nasal cavity to the CNS. The results of our study could improve the outcome for individuals affected by CNS-associated disorders by improving drug delivery via localized enhancement across the nasal epithelia.

Figure 2: Gemcitabine structure (*From PubChem Compound*)

HYPOTHESIS

Drug delivery to the brain is a challenge due to the presence of impenetrable BBB. Many of the CNS associated disorders, such as inoperable brain tumors and HIV associated dementia, cannot be treated effectively with orally administered drugs, as the chemotherapeutics cannot reach the brain in significant amounts. In recent times, the IN route has become popular for delivery of therapeutics due to its direct access to the brain. Many drugs have been successfully delivered to the brain via IN administration, but the goal of our study was to enhance IN delivery of our model nucleoside drug GEM, using a BBB permeabilizer PV. Our approach took advantage of the fact that the BBB and olfactory epithelial TJs share many proteins in common such as occludin, CLDN5 and ZO-1. We hypothesize that by transiently increasing the permeability of nasal epithelial TJs using the BBB permeabilizer PV, we will increase the concentration of GEM reaching the BECF following IN delivery, with the goal of delivering therapeutic concentrations of nucleoside drugs to the CNS.

SPECIFIC AIMS

- 1) (a) To determine the deposition of IN drugs/molecules.
(b) To determine safe IN dose(s) of PV and study the effect of IN PV on the target tissue, as well as distant organs.

- 2) (a) Assess the time course of altered levels and localization of TJ proteins in olfactory epithelia in response to IN PV treatment.
(b) To determine if PDE10A is expressed in the olfactory epithelium.

- 3) To determine whether IN pretreatment with PV will increase the delivery of IN GEM to the BECF in rats.

AIM 1 (a): To determine the deposition of IN drugs/molecules.

Introduction

Transport of drugs from the nasal cavity to the CNS occurs along olfactory and trigeminal nerve pathways, with olfactory nerve pathways are the major component of drug delivery from the nose to the brain (Jansson and Björk 2002). Studies with fluorescent tracers have shown a strong correlation between the concentrations of intranasal fluorescein dextran in the olfactory epithelium and olfactory bulb (Dhuria, Hanson, and Frey 2009). Rapid drug transfer from the olfactory region to the CNS has been previously demonstrated (John D. Hoekman and Ho 2011). Our study focuses on enhancing IN drug delivery to the CNS by increasing the paracellular permeability through olfactory epithelial TJs. The first step was to determine the distribution of IN drugs and confirm drug deposition in the olfactory region for direct transport to the CNS as shown by previous studies.

Materials and methods

Fluorescein isothiocyanate-dextran beads (FD4, average molecular weight 3000-5000) were obtained from Sigma Aldrich (St. Louis, MO) for the IN drug distribution study.

Animals

Male Sprague-Dawley rats (220-250 g) were obtained from (Harlan, Indianapolis, IN). The animals were housed individually in a room maintained at $22^{\circ}\text{C}\pm 1^{\circ}\text{C}$, $55\%\pm 10$ relative humidity with a 12-h/12-h light/dark cycle, and provided access to standard laboratory chow and tap water *ad libitum*. All procedures adhered to the “Principles of Laboratory Animal Care” guidelines and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Intranasal drug distribution study

FD4 beads were used to determine IN drug deposition. Rats were lightly anesthetized with ketamine/xylazine (87/13 mg/kg i.p.) and FD4 was administered intranasally (60 μL /nostril; 600 μg /animal, $n=4$) using sterile micropipette tips. Four h after IN FD4, animals were sacrificed. The following tissues were collected and stored at -80°C for fluorescence analysis: olfactory epithelium (OE) was collected from the ethmoid turbinates (ET); the maxilloturbinates (MT) and nasoturbinates (NT) were collected for nasal respiratory epithelium (RE); olfactory bulb (OB), forebrain (FB), hindbrain (HB) and cerebellum (CE) (**Figure 3**). Tissues were homogenized in protein lysis buffer (0.1 mM PMSF, 50 mM Tris HCl; pH 7.6, 2M urea, 0.1% NaCl, 0.1% EDTA and 0.1% Brij 35), centrifuged at $31,360\text{ g}$ (4°C) for 15 min and supernatant was collected. The Bradford assay was used to measure the protein concentration in the supernatants.

Spectrofluorometric analysis

The supernatants were heated for 10 min at 70°C and centrifuged at 20,070 *g* for 10 min to remove heme, as heme has the same excitation and emission wavelength as FD4. Clear supernatant was collected, and fluorescence was measured using a Hitachi F-4500 spectrofluorometer (EX=493nm, EM= 518nm, slit width- 5, 20). In order to correct for potential tissue auto fluorescence, the corresponding tissues were assayed from rats not treated with FD4. Values obtained from the untreated animals, while negligible, were subtracted from the fluorescence values for the FD4-treated rats.

Statistical analysis

ANOVA (analysis of variance) was used to measure the statistical differences in fluorescence in different tissues. Multiple comparisons were carried out using Tukey's honest significant difference method.

Figure 3: **Rat nasal cavity structure** showing the maxilloturbinates (MT) and naso-turbinates (NT), ethmoid turbinates (ET) and olfactory bulb (OB).

Results

In order to determine the distribution of intranasally administered materials using our procedures; IN drug distribution was evaluated using fluorescent beads (FD4). The results show significant fluorescence, attributable to the FD4 beads, in the OE as compared to other tissues, thus suggesting that drug delivery to the CNS from the nasal cavity occurs through the OE (**Figure 4**).

Figure 4: Concentration (Fluorescent units/ μg of protein, Mean $\pm$ SE) of FD4 in olfactory epithelium (OE), olfactory bulb (OB), respiratory epithelium (RE), hindbrain (HB), cerebellum (CE) and forebrain (FB) 4hr after IN administration of FD4 (5mg/ml). *Significantly different from CE at $p < 0.001$ ($n=4$).

AIM 1 (b): To determine safe IN dose(s) of PV and study the effect of IN PV on the target tissue, as well as distant organs.

Introduction

Papaverine hydrochloride is an opium alkaloid and is used as a vasodilator and smooth muscle relaxant (Boswell-Smith, Spina, and Page 2006) but this compound differs in both chemical structure and therapeutic effects from other related opium alkaloids. PV undergoes biotransformation in the liver by O-demethylation and subsequent conjugation and the polar metabolites are excreted in bile (Belpaire and Bogaert 1975). Mephenytoin p-hydroxylating cytochrome P-450 (CY2C19) and sparteine-oxidizing cytochrome P-450 (CYP2D6) enzymes that are mainly localized in liver and kidney, have been found to be involved in PV metabolites formation (Belpaire and Bogaert 1975; Ebner, Meese, and Eichelbaum 1995; Inaba et al. 1985; Yuan et al. 2002). CYP2C enzyme expression has been shown in nasal tissues, so local metabolism of IN PV is also possible (X. Ding and Kaminsky 2003). PV has been used for treating a range of diseases, including cerebral and coronary artery vasospasm (Liu and Couldwell 2005; Sivalingam, Levine, and Dunning 2005), acute renal colic (Yencilek et al. 2008), and erectile dysfunction (Kirby 1994; Mooradian et al. 1989). PV is an inhibitor of PDE 10A, which is localized primarily in the brain regions (Seeger et al. 2003). The exact mechanism of action of PV is still unclear but its putative mechanism is to elevate cAMP via the inhibition of PDE. In recent times, PV is gaining attention for its potential in the treatment of

neuropsychiatric diseases such as schizophrenia. PV has shown antipsychotic activity in animal models of schizophrenia (Siuciak et al. 2006). Many studies have demonstrated the role of PV in treatment of cerebral vasospasm, and different intra-arterial doses of PV have been tested to check for their efficacy and any associated toxicity (Platz et al. 2008; Yoshimura et al. 1996; Sawada et al. 1997). PV doses for this study were selected on the basis of previous studies showing transient increases in BBB and blood tumor barrier (BTB) permeability after PV infusion (A K Bhattacharjee et al. 2001; Z.-H. Wang, Xue, and Liu 2010; Platz et al. 2008; Sawada et al. 1997; Yoshimura et al. 1996). Keeping in consideration the limitation with IN drug volume and PV solubility, we selected 3 clinically relevant doses of PV (0.3%, 0.8% and 1.4%) to determine if these doses could be administered without causing toxicity to the nasal epithelia or distant organs.

Chemicals and supplies

Papaverine hydrochloride was obtained from Fischer Scientific (Pittsburg, PA).

Animals

Male Sprague-Dawley rats (220-250 g) were obtained from (Harlan, Indianapolis, IN). The animals were housed individually in a room maintained at $22^{\circ}\text{C}\pm 1^{\circ}\text{C}$, $55\%\pm 10$ relative humidity with a 12-h/12-h light/dark cycle, and provided access to standard laboratory chow and tap water *ad libitum*. All procedures adhered to the “Principles of Laboratory Animal Care” guidelines and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Histology

In order to determine whether PV treatment caused damage to nasal epithelia, PV was administered for 5 consecutive days (0.3, 0.8, or 1.4% in left nostril; 60 μ L/nostril) and rats were sacrificed (carbon dioxide asphyxiation) for histological examination. The untreated right nostril served as the control tissue. Nasal cavities were fixed in 10% neutral buffered formalin and decalcified in 10% formic acid prior to paraffin embedding. Sections (5 microns in thickness), taken at standard levels of the nasal cavity (St. Clair and Morgan 1992), were stained with hematoxylin and eosin (H&E). Another group of animals was treated with a single dose of PV (1.4%; 60 μ L in left nostril) and sacrificed 1 d or 5 d later for examination of other tissues for potential toxicity induced by PV. Nasal cavities, liver, kidney, spleen and lungs were collected, fixed in formalin, and embedded in paraffin. Nasal cavities were decalcified in formic acid for 7 days, and then rinsed overnight with tap water. Lungs were fixed by inflation with formalin (30 cm height). Sections (5 microns) were stained with H&E and examined by light microscopy.

Immunohistochemistry

Olfactory marker protein (OMP) is a marker of mature olfactory neurons and their axons. OMP immunohistochemistry (antibody was the generous gift of Dr. Frank Margolis, University of Maryland) was conducted as described previously (Young 1981). Immunolocalization was detected using peroxidase Goat IgG vectastain

ABC kit (Vector Labs, Burlingame, CA). Immunostained sections were counterstained with hematoxylin and photographed with a Nikon D80 camera.

Results

PV treatment (once/d for 5 consecutive d) did not cause histological damage to the OE (**Figure 5**). In addition, IN PV did not cause a decrease in immunohistochemically-detectable OMP (a marker of mature olfactory neurons and their axons) at concentrations of up to 1.4% for up to 5 d (**Figure 6**), as evidenced by robust staining on both the untreated and treated sides of the nasal cavity and in the nerve bundles of the corresponding olfactory bulb sections. Histological examination of nasal epithelia at the end of 8 h sample collection period identified no epithelial damage as a result of PV and GEM treatment (not shown). Examination of lung, liver, kidney, and spleen from rats treated with a single dose of 1.4% PV and sacrificed 1 or 5 days later showed no evidence of inflammation, hemorrhage, or toxicity (**Figure 7**).

Figure 5: Frontal nasal cavity section showing no damage to nasal epithelia comparing the 1.4% PV-treated (**) and untreated sides of the nose. NS=nasal septum.

Figure 6: Olfactory marker protein (OMP) staining, showing normal staining in the treated side of nasal cavity (*) of a rat treated with 0.8% PV (left) and in the olfactory bulb (right) corresponding to the treated side of the nasal cavity (*). High power photomicrograph (middle) shows normal cellular structure and OMP immunoreactivity in the epithelium (OE) and in subepithelial nerve bundles (NB).

Figure 7: Photomicrographs from rats receiving a single IN PV treatment (1.4%) and sacrificed either 1d or 5d after treatment. Note lack of inflammation, hemorrhage, or toxicity in lung, liver, spleen, and kidney. Sections were stained with hematoxylin and eosin and photographed at 10X magnification.

AIM 2 (a): Assess the time course of altered levels and localization of TJ proteins in olfactory epithelia in response to IN PV treatment.

Introduction

Previous studies with PV have shown transient increase in BBB and BTB permeability after PV infusion and it was found to be associated with downregulation of BBB proteins (Z.-H. Wang, Xue, and Liu 2010; Abesh Kumar Bhattacharjee et al. 2002; Platz et al. 2008). Olfactory epithelial TJs express many proteins also localized to the BBB, including ZO-1, occludin, and CLDN5 (Wolburg et al. 2008). Because of the similarity in structure between the BBB and olfactory epithelial TJs, we propose that a strategy that has been demonstrated to be effective in transiently increasing permeability of the BBB will also transiently perturb olfactory epithelial TJs, thereby enhancing the delivery of IN-administered drugs to the CNS. In order to study the mechanism of action of IN PV on olfactory TJ proteins, a time course study was designed to measure the levels and localization of the TJ proteins occludin and claudin 5 after IN PV treatment.

Materials

Papaverine hydrochloride was obtained from Fischer Scientific (Pittsburg, PA).

Animals

Male Sprague-Dawley rats (220-250 g) were obtained from (Harlan, Indianapolis, IN). The animals were housed individually in a room maintained at $22^{\circ}\text{C}\pm 1^{\circ}\text{C}$,

55±10% relative humidity with a 12-h/12-h light/dark cycle, and provided access to standard laboratory chow and tap water *ad libitum*. All procedures adhered to the “Principles of Laboratory Animal Care” guidelines and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Methods

Western blot analysis

Western blot analysis was performed to investigate the expressions of occludin and CLDN5 at different time points after PV administration. Rats were treated with 1.4% PV (60 µL in each nostril) and sacrificed 0, 15, 30, 60 and 120 min after PV treatment. OE from the ethmoid turbinates and caudal one-third of the nasal septum was immediately frozen on dry ice, and stored at -80°C. Tissues were homogenized in protein lysis buffer (0.1 mM PMSF, 50 mM Tris HCl; pH 7.6, 2M urea, 0.1% NaCl, 0.1% EDTA and 0.1% Brij 35), centrifuged at 31,360 *g* (4°C) for 15 min and supernatant was collected. The Bradford assay was used to measure the protein concentration in collected supernatants. Equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting with antibodies for occludin (1.25µg/ml, Invitrogen) and CLDN5 (2.5µg/ml, Invitrogen). Membranes were stained with Ponceau Red (Sigma, St. Louis, MO USA) to assure equal protein loading. β-Actin (diluted 1:10,000, Santa Cruz Biotechnology, Inc.) was used as an internal control. Immune complexes were visualized using the enhanced chemiluminescence (Pierce ECL Western blotting Substrate, Thermo Scientific). The protein bands

were scanned using Epson perfection V750 Pro scanner, and band densities were calculated by ImageJ software (<http://rsbweb.nih.gov/ij/>) and normalized with that of β -actin.

***In vitro* PV incubation with OE homogenates and dot blot analysis**

Upon noting the apparent decrease in occludin protein by western blot analysis (discussion section), we sought to determine whether the ability of the anti-occludin antibody to detect the antigen was altered in the presence of PV, suggesting a possible conformational change of the occludin protein by PV. OE homogenate was prepared from untreated rats and protein concentration determined as discussed above. Equal amounts of OE homogenate (20 μ g) were incubated with 1.4% PV for different time points (0, 15, 30, 60, and 120 min) on ice. After *in-vitro* incubation, the homogenates were blotted on a nitrocellulose membrane under non-reducing and non-denaturing conditions, blocked with BSA and incubated with anti-occludin antibody (1.25 μ g/ml, Invitrogen) at 4°C overnight. Immune complexes were visualized using the enhanced chemiluminescence (Pierce ECL Western blotting Substrate, Thermo Scientific).

Immunofluorescence localization of occludin following PV treatment

In order to determine whether IN PV treatment alters the immunolocalization of the nasal epithelial TJ protein occludin, PV was administered IN (1.4% PV; 60 μ L/nostril) and rats were sacrificed (carbon dioxide asphyxiation) after 0, 15, 30, 60 and 120 min. Nasal cavities were fixed in 10% neutral buffered formalin and

decalcified in 10% formic acid prior to paraffin embedding. Sections (5 microns in thickness) were taken at standard levels of the nasal cavity (St. Clair and Morgan 1992). Anti-occludin antibody (1:400, Santa Cruz Biotechnology Inc.) was incubated with the nasal section slides overnight at 4°C. The slides were then incubated with secondary antibody Alexa Fluor 488 Donkey anti-rabbit IgG (Invitrogen, 1:200) for 2 h at room temperature in dark. The slides were mounted with a coverslip using Vectashield HardSet Mounting Medium with DAPI (Vector; H-1500). Nasal sections were photographed individually with a Leica TCS SP Spectral Confocal microscope.

Statistical analysis

ANOVA was used to determine the statistical significant differences in the levels of nasal epithelial TJ proteins (occludin and claudin-5) separately at five different time points. Multiple comparisons were carried out separately for occludin and claudin-5 using Tukey's honest significant difference method.

Results

Response of nasal epithelial TJ proteins to IN PV treatment: Levels of the phosphorylated form of occludin and claudin-5 were determined by western blot analysis. After IN PV treatment (1.4%, 60µL/nostril), phosphorylated occludin immunoreactivity in OE was decreased by 15 min. The lowest level appeared at 30 min and increased thereafter. In the case of phosphorylated occludin, the five different time points showed significant differences ($p < 0.05$). The control (time=0

min) was significantly different from each of the time points t=30 min, 15 min and 60 min. The relative density ratio of occludin (compared to β -actin) is shown in **(Figure 8)**. There was no significant change in the expression levels of CLDN5 at any time point ($p=0.59$) **(Figure 9)**.

Results of dot blot analysis of olfactory epithelial homogenates treated *in vitro* with 1.4% PV indicate that PV treatment reversibly alters the conformation of the protein such that the antibody does not recognize it **(Figure 10)**.

Immunofluorescence localization of occludin following IN PV treatment: **Figure 11** shows the results of immunofluorescence analysis of occludin localization in the OE in a control rat (left), a rat 30 min after IN treatment with PV (middle), and a rat 120 min after IN PV treatment (right). Note that in the control rat and in the rat treated 120 min earlier, occludin appears as sharp crisp green bands along the edges of the apical surface of olfactory epithelial cells, whereas in the rat treated 30 min previously with PV, staining is much less intense and less discrete, with diffuse staining throughout the apical cytoplasm of the olfactory epithelial cells, suggesting that PV treatment alters the localization and/or conformation of occludin.

Figure 8: Western blot analysis showed that IN PV treatment (1.4%) induced a transient decrease in levels of phosphorylated occludin (65~79 KDa) in OE. From left to right, the lanes are control (0 min), 15 min, 30 min, 60 min and 120 min. Relative density ratio (Mean± SE) of phosphorylated occludin/β-actin at different time points (t=0 min, 15 min, 30 min, 60 min and 120 min; n=3). **Significantly different from t=0 min at p<0.05.

Figure 9: Relative density ratio (Mean± SE) of claudin-5/β-actin at different time points (t=0 min, 15 min, 30 min, 60 min and 120 min; n=3). No significant change was seen in the protein levels of claudin-5 (CLDN5).

Figure 10: Dot blot analysis of olfactory epithelial homogenates treated *in vitro* with 1.4% PV. Results indicate that PV treatment reversibly alters the conformation of the protein such that it is not recognized by the antibody.

Figure 11: Occludin Immunofluorescence

Occludin appears as sharp crisp green bands (yellow arrows) along the edges of the apical surface of olfactory epithelium in control rat (left) and a rat 120 min (right) after IN PV treatment (1.4%). In the rat treated 30 min (middle) previously with IN PV (1.4%), staining is less discrete and diffused throughout the apical cytoplasm (**) of the olfactory epithelial cells, suggesting that PV treatment reversibly alters localization or conformation of occludin. Sections were photographed at 400X magnification.

AIM 2 (b): To determine if PDE10A is expressed in the olfactory epithelium.

Introduction

PV is a specific inhibitor of PDE10A, which is localized in the striatum, testis, epididymal sperm, and enteric ganglia (Fujishige et al. 1999; Fujishige, Kotera, and Omori 1999; Coskran et al. 2006). PDE10A expression in OE has never been studied before. To determine if PDE10A is expressed in OE, we did a reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Results of PDE10A expression study helped us understand if PV's action is limited to the olfactory epithelial TJs or if it also acts on PDE10A present in the OE.

Animals

Male Sprague-Dawley rats (220-250 g) were obtained from (Harlan, Indianapolis, IN). The animals were housed individually in a room maintained at 22°C±1°C, 55±10% relative humidity with a 12-h/12-h light/dark cycle, and provided access to standard laboratory chow and tap water *ad libitum*. All procedures adhered to the "Principles of Laboratory Animal Care" guidelines and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

RNA isolation, cDNA synthesis and PDE10A RT-PCR

Rats were sacrificed by decapitation. Striatum, OE and OB were collected from untreated control rats, immediately frozen on dry ice and stored at -80°C until RNA isolation. RNA was isolated using Tri Reagent (Molecular Research Center,

Cincinnati, OH) according to the manufacturers' protocol. Total RNA quantity and quality were assessed by absorbance ($_{260\text{ nm}}$) and absorbance ratio of 260/280 nm utilizing Agilent Bioanalyzer/Nanodrop analysis (Agilent 2100 Bioanalyzer). RNA samples with absorbance ($_{260/280}$) ratios 1.95–2.0 were used for qRT-PCR. cDNA synthesis was performed using an RNA-to-cDNA kit (Verso cDNA synthesis kit, #AB1453, Thermo Fisher Scientific, Waltham, MA). The following PCR primers for rat PDE10A gene were used: sense 5'- TGCTTCCGAAGGCTGACCGA-3' and antisense 5'-GCACTCTCCAAGGAAG- TACA-3', which amplified a 360-bp fragment (Giorgi et al. 2011). Forty PCR cycles were performed on cDNA after an initial denaturing step of 4 min at 94 °C using the following profile: 94 °C for 1 min; 60 °C for 2 min; 72 °C for 3 min, including a final extension of 7 min at 72 °C. The PCR products were separated by electrophoresis on a 2% agarose gel and detected with ethidium bromide staining.

Results

Figure 12 shows a gel picture with PCR products from striatum (S), OE and OB. Sharp bands were observed in striatum (positive control) and OB indicating PDE10A expression in these tissues. No band was observed in OE, suggesting no PDE10A expression in OE.

Figure 12: Gel picture showing PDE10A RT-PCR products. Lanes from left to right- L (Ladder), B (blank-no cDNA), S (striatum), OE (olfactory epithelium) and OB (olfactory bulb).

AIM 3: To determine whether IN pretreatment with PV will increase the delivery of IN GEM to the BECF in rats.

Introduction

Clinically relevant doses of IN PV- 0.3%, 0.8% and 1.4 % did not cause histological damage in nasal epithelia, lung, liver, kidney and spleen. Previous studies with PV have shown transient increase in BBB and BTB permeability after PV infusion and it was found to be associated with downregulation of BBB proteins (Z.-H. Wang, Xue, and Liu 2010; Abesh Kumar Bhattacharjee et al. 2002; Platz et al. 2008)). Due to similarity between olfactory epithelial TJ proteins and BBB proteins and transient decrease in levels of phospho-occludin upon treatment with IN PV, we hypothesized that PV will increase the delivery of our model drug GEM to the brain by transiently increasing paracellular transport through olfactory epithelial TJs (Wolburg et al. 2008).

A non-toxic dose of IN PV (1.4%) resulted in transient decrease in the levels of olfactory epithelial TJ protein, phospho-occludin. The next step was to determine the efficacy of IN PV in increasing the concentration of GEM reaching the BECF following IN administration. BECF samples were collected for 8 h, every 30 min and analyzed by HPLC to determine the effectiveness of PV in enhancing the delivery of GEM to the brain.

Brain microdialysis

Unbound drug concentrations in the brain extracellular fluid (ECF) provide information about tissue exposure to the drug and are important determinant of pharmacological effect (Plock and Kloft 2005). ECF is viewed as the region in the immediate vicinity of cells where drug must enter. Exposure of tumor cells or the diseased region in the brain to free drug in the BECF can be considered comparable to that observed *in vitro* in cell cultures, thus monitoring drug concentrations in ECF is an important factor in optimizing the drug dosage and understanding the time course of drug effects (Fenstermacher, Patlak, and Blasberg 1974; E. C. de Lange 2013; Darvesh et al. 2011).

In vivo brain microdialysis was invented in the 1970's by Ungerstedt in order to study release of neurotransmitters (Ungerstedt and Pycock 1974). In recent times, microdialysis has become very useful *in vivo* and *in vitro* tool for a variety of pharmacological and metabolic applications (Plock and Kloft 2005). It has been demonstrated that with *in vivo* brain microdialysis, BBB transport characteristics of drugs, as well as local concentration differences within the brain regions can be studied.

Gemcitabine (GEM)

Gemcitabine (2', 2'-difluorodeoxycytidine; dFdC) hydrochloride (MW 263.19) is a deoxycytidine analog and is used as a chemotherapeutic agent in the treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer and breast

cancer (Noble and Goa 1997). This compound was initially used as an anti-viral agent but its structural and metabolic similarity with anticancer drug cytarabine made it a potential candidate for the treatment tumors (Hertel et al. 1990). This drug was approved by U.S. Food and Drug administration in 1996 for treating advanced and metastatic pancreatic cancer. Recent studies have demonstrated its efficacy in the treatment of glioblastomas (Sigmond et al. 2009; Apparaju, Gudelsky, and Desai 2008; Gertler et al. 2000; Wick et al. 2002; Degen et al. 2003). In our study we are using GEM as a model nucleoside drug for enhanced IN delivery into the BECF.

Materials

Gemcitabine (2', 2'-difluorodeoxycytidine) hydrochloride was obtained from Enzo Life Sciences (Farmingdale, NY). The drug was reconstituted for IN administration in sterile 0.9% sodium chloride to a final concentration of 150 mg/mL. Papaverine hydrochloride was obtained from Fischer Scientific (Pittsburg, PA). Tetrahydrouridine, a deoxycytidine deaminase inhibitor, was obtained from Calbiochem (San Diego, CA). Cannulation materials for the implantation were purchased from Plastics one Inc. (Roanoke, VA) and VWR Scientific (Batavia, IL). Dialysis membrane with a 13,000 MW cutoff and an outside diameter of 210- μ m was purchased from Spectrum Laboratories (Rancho Dominguez, CA). HPLC grade solvents were obtained from Fisher Scientific and VWR International. Concentric-style microdialysis probes were constructed as described (Yamamoto and Pehek 1990).

Animals

Male Sprague-Dawley rats (220-250 g) were obtained from (Harlan, Indianapolis, IN). The animals were housed individually in a room maintained at 22°C±1°C, 55±10% relative humidity with a 12-h/12-h light/dark cycle, and provided access to standard laboratory chow and tap water *ad libitum*. All procedures adhered to the “Principles of Laboratory Animal Care” guidelines and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Methods

In vitro recovery

In order to relate the concentrations in the dialysate to the true concentrations in the tissue outside the probe and to measure the extent to which the compound of interest is recovered by the dialysate, *in vitro* recovery was done. Recovery was determined with the probe in aqueous solutions containing 20 mM, 50 mM or 63.3 mM GEM. The probe was then continuously perfused at a constant flow rate with dialysis fluid (Dulbecco's phosphate-buffered saline containing 1.2 mM CaCl₂ and 5 mM glucose) devoid of GEM, and samples were collected during fixed time intervals. The percent recovery of the solute was then determined as follows:

% Recovery *in vitro* = $[C_{\text{dial}}/C_{\text{bulk}}] \times 100$, where C_{dial} is the concentration of GEM in the dialysate sample and C_{bulk} is the concentration of GEM in the bulk reservoir.

Cannulation procedure

Rats were anesthetized with an injection of ketamine and xylazine (87/13 mg/kg, i.p.). The coordinates for the striatum of the brain were identified according to the atlas of Paxinos and Watson (1.2 mm anteroposterior and $\pm$ 3.1 mm lateral from bregma) and a hole was drilled through the cranium, dorsal to the striatum (Paxinos and Watson). A guide cannula was then lowered into the hole and secured to the skull with screws and cranioplastic cement. Animals were allowed to recover for 3 d before the drug administration and sampling.

PV administration and sampling protocol

On the morning of the experiment, concentric style microdialysis probes were inserted through the guide cannulae. The length of the dialysis membrane for the striatum was 2.5 mm. The probes were perfused with simulated brain interstitial fluid (Dulbecco's phosphate-buffered saline containing 1.2 mM CaCl_2 and 5 mM glucose) at an input rate of 1.8 $\mu\text{L}/\text{min}$, in order to facilitate an optimal temporal resolution and higher recovery of GEM. Probes were perfused and allowed to equilibrate for 1.5 h before initiating drug administration and sampling procedures.

Before administering drugs intranasally, the rats were anesthetized with ketamine/xylazine (87/13 mg/kg, i.p.). PV solutions [3 mg/ml (0.3%), 8mg/ml (0.8%) and 14mg/ml (1.4%)] were prepared in 0.9% saline. PV was administered intranasally at 60 μL / nostril in each nostril and 15 min after PV treatment; GEM (50 mg/kg; also dissolved in sterile saline) was administered intranasally

(n=4/treatment group). GEM dose was selected on the basis of previous studies (Apparaju, Gudelsky, and Desai 2008; Degen et al. 2003). A control group of animals (n=4) received saline infusion instead of PV, followed by GEM.

Dialysate samples were collected in tubes containing the cytidine deaminase inhibitor tetrahydrouridine (0.5 mM final concentration) every 30 min after GEM treatment for 8 h.

HPLC analysis of GEM in dialysate

The analysis of GEM was done using a slight modification of a published procedure (L. A. Shipley et al. 1992). The mobile phase consisted of ammonium acetate:acetonitrile (98:2, v/v) buffer (0.1 M) [with acetic acid (0.05 %) and triethylamine to adjust the pH to 7.0]. The mobile phase was pumped at a flow rate of 1 mL/min using a Waters 510 pump. GEM was eluted on a Waters Nova-Pak® C18 4µm column coupled with a guard column of similar properties. A Waters 486 variable wavelength UV/VIS detector coupled to millennium software was used for detecting and integrating the sample peaks. The lower limit of quantitation for GEM using this method of analysis was 25 ng/ml for the microdialysate samples. The intra- and inter-day precision of the analyses was < 15 %. The accuracy was within ± 12 %.

BECF distribution of GEM and data analysis

Non-compartmental analysis of GEM concentration versus time data in BECF was performed employing WinNonlin version 6.1, (Pharsight, CA). The BECF

concentrations of GEM were time-averaged over the dialysate collection interval. The pharmacokinetic parameter, area under the curve (AUC) values of GEM in BECF was calculated employing the linear trapezoidal rule, where the total area under the drug concentration versus time curve was measured by summing up a number of incremental areas from each trapezoid.

$AUC_{0-t_n} = \sum_{i=1}^n [(C_i + C_{i+1})/2] \times \Delta t$, where, C_i and C_{i+1} are the concentrations at time points t_i and t_{i+1} , respectively and the time interval, $\Delta t = (t_{i+1} - t_i)$.

Since the sampling interval of 30 min employed in our study may not facilitate temporal resolution required for precise determination of elimination half-life, only AUC_{0-t} , where t is the time corresponding to the last sample collection, was determined.

Statistical analysis

ANOVA was used to determine the statistical differences. Multiple comparisons were carried out using Tukey's honest significant difference method.

Figure 13: Experimental Setup

Results

In vitro GEM recovery: *In vitro* recovery of GEM was calculated to be $12.73 \pm 1.04\%$. The percent recovery was same with three different concentrations of GEM (20 mM, 50 mM or 63.3 mM).

PV enhancement of GEM delivery to BECF: While 0.3% PV did not appreciably enhance GEM delivery to BECF, 1.4% PV treatment significantly increased delivery of GEM to BECF (**Figure 14**). The AUC for GEM in 1.4% PV treatment ($5.54 \pm 0.83 \mu\text{g}\cdot\text{hr}/\text{ml}$) shows almost four fold increase as compared to 0% PV treatment ($1.5 \pm 0.28 \mu\text{g}\cdot\text{hr}/\text{ml}$) group. A similar increase in presence of PV was also observed for GEM C_{max} within the BECF samples. Mean $\pm$ SD C_{max} values were 0.35 ± 0.16 , 0.41 ± 0.29 , 0.6 ± 0.09 , and $2.3 \pm 1.6 \mu\text{g}/\text{ml}$, respectively at PV doses of 0%, 0.3%, 0.8% and 1.4%. There appeared to be a dose-related increase in the uptake of GEM with PV pre-treatment. The T_{max} for 1.4% PV group was 0.5 hr compared to 1 hr for 0% PV group. This indicates that the T_{max} was attained at an earlier time point on treatment with PV when compared with no PV group. For all treatment groups, GEM levels in BECF started returning to the baseline after 2-2.5 hr.

Figure 14: Effect of IN PV on GEM AUC in BECF.

Relative changes in the AUC values (Mean $\pm$ SE) of GEM in rat BECF on treatment with different doses of PV (0%, 0.3%, 0.8% and 1.4%; n=4/ treatment group) *p $\leq$ 0.05 and **p<0.01.

DISCUSSION

The current study sought to tie together several important observations. First, the potential to deliver anti-HIV nucleoside drugs to the CNS following IN administration in rodents was shown nearly two decades ago (T Seki et al. 1994). One means by which nucleoside drugs are taken up is by the equilibrative nucleoside transporters, and we have previously demonstrated the presence of two equilibrative nucleoside transporters (*SLC29A1* and *SLC29A2* gene, formerly ENT1 and ENT2) on the surface of the olfactory mucosa (Genter et al. 2010). However, careful review of the literature does not suggest a strategy that might be effective in increasing the activity or expression of these transporters. Second, very recent clinical reports indicate that IN administration of a Ras inhibitor (monoterpene perillyl alcohol) was effective in extending the life span of human patients with end stage glioblastoma (da Fonseca et al. 2011), indicating the importance of this route of drug delivery for serious, intractable CNS disorders.

Because many of the same proteins are expressed in the BBB and in olfactory epithelial TJs (Wolburg and Lippoldt 2002; Wolburg et al. 2008), and PV has previously shown to transiently increase the permeability of the BBB and BTB by altering the levels of cytoskeletal and TJ-associated proteins (A K Bhattacharjee et al. 2001; Platz et al. 2008; Z.-H. Wang, Xue, and Liu 2010; H. Xie et al. 2010; Abesh Kumar Bhattacharjee et al. 2002) , we hypothesized that PV would have a beneficial effect on drug delivery to CNS by reversibly increasing the permeability of olfactory epithelial TJs.

Drugs or molecules can be transported from the nose to the brain by two possible routes, transneuronal and paracellular transport (Frey II 2002; Thorne et al. 2004; Dhanda et al. 2005; W. Pardridge 2005). Transporter-mediated drug uptake involves a balance between efflux transporters such as MRPs 1, 3, 4, and 5, P-glycoprotein and BCRP as well as influx transporters such as ENT1 and 2; many of these transporters have been localized in the rat olfactory epithelium (Genter et al. 2010; Molinas, Sicard, and Jakob 2012; Kudo, Doi, and Fujimoto 2010; Thiebaud et al. 2010; Agarwal et al. 2013). Studies have shown GEM transport is mediated by hENT1, and rat ENT1 mediates GEM translocation across the BBB (Farrell et al. 2009; Thomas F.C. 2008). However, active efflux limits net GEM translocation, thus resulting in overall low transporter-mediated GEM influx into brain (Thomas F.C. 2008). Our goal was to enhance IN drug delivery to the brain, and since we do not know of a mechanism to upregulate or increase the expression of drug transporters in the nasal epithelia, we decided to target the paracellular route by increasing the permeability through nasal epithelial TJs.

The observation that IN-administered materials were extensively delivered to the olfactory region of the nasal cavity, coupled with the observation that peak GEM concentrations in BECF were observed earlier with IN PV treatment (30 min) compared to the peak concentration at 60 min without PV, strongly suggest that drug delivery to the brain occurs across the OE. GEM, our model drug, is a small

(MW 299.66) polar molecule and has 7% plasma protein binding ratios in rat and dog and 10% in human (Esumi et al. 1994). Since it is a small hydrophilic molecule, it can be transported across the nasal epithelial TJs by paracellular transport.

In the current study, PV treatment enhanced GEM delivery to the brain in a dose-dependent manner without causing any evidence of toxicity to nasal epithelia or distant organs, even with repeated exposure. Importantly, IN-administered GEM + PV resulted in GEM levels in BECF that were comparable to those attained following intravenous administration of GEM together with another BBB permeabilizer, RMP-7 (Apparaju, Gudelsky, and Desai 2008). Therefore, this non-toxic and non-invasive method might prove to be useful for enhancing the delivery of other IN-administered drugs to the CNS. Other phosphodiesterase inhibitors such as sildenafil (Viagra®) and vardenafil (Levitra®) have been found to increase herceptin transport and treatment efficacy in mouse metastatic brain tumor models when orally administered (Hu et al. 2010).

In order to understand the mechanism and time course by which PV exerts its beneficial effect on IN drug delivery, the levels of key olfactory epithelial TJ proteins were assessed using western blot analysis. We observed a rapid and transient decrease in level of the nasal epithelial TJ protein occludin (phosphorylated form) after IN PV treatment, with no change in the expression of claudin-5 over the same time course (Z.-H. Wang, Xue, and Liu 2010; H. Xie et

al. 2010). The results of dot blot analysis indicate that the transient decrease in levels of occludin after IN PV treatment could be due to the failure of the antibody to recognize the PV-modified occludin. The rapid and transient increase in paracellular permeability across nasal epithelial TJs can be explained by transient dephosphorylation of occludin leading to TJ disassembly into the cytoplasm (Aim 2; **Figure 11**). It has been previously demonstrated that transient increase in *in vivo* nasal absorption of FITC-dextran after poly-L-arginine treatment is due to enhanced paracellular permeability via serine/threonine phosphorylation of ZO-1 and tyrosine dephosphorylation of occludin (Ohtake et al. 2003; Ohtake et al. 2002). **Figure 11** (Aim 2) shows the results of immunofluorescence analysis of occludin localization in the olfactory epithelium in a control rat (left), a rat 30 min after IN treatment with PV (middle), and a rat 120 min after IN PV treatment (right). Note that in the control rat and in the rat treated 120 min earlier, occludin appears as sharp crisp green bands along the edges of the apical surface of olfactory epithelial cells, whereas in the rat treated 30 min previously with PV, staining is much less intense and less discrete, with diffuse staining throughout the apical cytoplasm of the olfactory epithelial cells. We interpret this finding to represent transient TJ disassembly into the cytoplasm due to transient dephosphorylation or altered conformation of occludin in the olfactory epithelium 30 min following PV treatment, with a return to the normal pattern of expression 120 min after treatment. Both of these observations are consistent with the known biological half-life of PV, 1.5-2.2 hr (Ritschel and Hammer 1977). Possible mechanism of action for effect of PV on occludin in OE

could be inactivation of kinases or activation of phosphatases involved in transient occludin dephosphorylation in the OE. PDE10A RT-PCR analysis showed no PDE10A expression in the rat OE, thus eliminating the possibility of PDE10A inhibition in OE following IN PV treatment as a mechanism of increasing drug delivery. Our results suggest that IN PV treatment enhances paracellular permeability across nasal epithelial TJs by transient dephosphorylation or altered conformation of occludin, thereby delivering significant concentration of our model drug GEM to the BECF.

We are targeting the olfactory region of the nose for enhancing the delivery for our model drug, GEM, to the brain and our hypothesis and results suggest that the site of action of PV is OE. The OE surface area in rats and humans is 7 cm² and 10 cm² respectively (Pires et al. 2009). Using pressurized olfactory delivery (POD) devices, significant amount of drug can be deposited in the olfactory region for direct delivery to the brain (Hoeman and Ho 2012). In order to extrapolate and calculate an equivalent IN PV dose in humans we have to keep into consideration the interspecies difference in the OE surface area. Rodents are obligate breathers and 50% of their nasal cavity is covered with OE (7 cm²) compared to just 3-10% (10 cm²) in humans (Harkema, Carey, and Wagner 2006). The efficacy of IN drug delivery to the brain can be improved by targeting drug deposition in the OE using POD devices. Assuming POD device is used for IN PV delivery to the OE in humans, we can calculate the IN PV dose that will be required for increasing paracellular permeability through olfactory epithelial TJs,

which are present in 10 cm² of OE in humans. In our study, for 7 cm² of OE in rat, 1.4% IN PV (1.68 mg PV/rat) increased the amount of IN GEM reaching the BECF (compared to no PV treatment) by transiently altering the levels and localization of olfactory epithelial TJ protein occludin. For 10 cm² of OE in humans, approximately 2.4 mg/person IN PV dose might be able to enhance IN drug delivery to the brain.

Thus, the IN route, coupled with a non-toxic, reversible epithelial tight junction permeabilizer such as PV, represents a viable means to non-invasively deliver nucleoside drugs to the CNS. This strategy could dramatically improve treatment of brain tumors and CNS HIV/AIDS infections.

FUTURE STUDIES: Since we have not examined whether sufficient PV gets in to the CNS to affect global BBB permeability, the next step would be to determine the effect of IN PV on whole BBB permeability. Also, we have not shown the effectiveness on IN PV+GEM in treatment of a CNS disorder such as brain tumors. Our long-term goal is to determine the efficacy of IN PV+GEM in the treatment of implanted brain tumors in rats.

CONCLUSIONS

A non-toxic dose of IN PV (1.4%) transiently decreased the levels of olfactory epithelial TJ protein, p-occludin and reversibly altered the localization of occludin in rat OE. The ability of IN PV to enhance the delivery of IN GEM was demonstrated by almost four fold increase in AUC for GEM in BECF in PV (1.4%) + GEM (50mg/kg) treatment group as compared to no PV group. Thus, it appears that transient permeabilization of olfactory epithelial TJs using PV provides a non-invasive means to enhance IN delivery of GEM to the brain.

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CHAPTER 2

Toxicology and Risk Assessment of Chemical Mixtures

LIST OF ABBREVIATIONS

ATSDR	Agency for Toxic Substances and Disease Registry
CAG	Cumulative Assessment Group
CIRS	Chemical Inspection and Regulation Service
CMG	Common Mechanism Group
EFSA	European Food Safety Authority
FEMA	Flavor and Extract Manufacturers Association
HI	Hazard Index
ILSI- HESI	International Life Sciences Institute- Health and Environmental Sciences Institute
JECFA	Joint Expert Committee on Food Additives
MOA	Mode of Action
NIEHS	National Institute of Environmental Health Science
NIOSH	National Institute for Occupational Safety and Health
NIS	Sodium/Iodide Symporter
NOAEL	No-observed-adverse-effect- level
OECD	Organization for Economic Co-operation and Development

PAH	Polyaromatic Hydrocarbons
RfC	Reference Concentration
RfD	Reference Dose
RFPs	Relative Potency Factors
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Community on Health and Environmental Risk
TNO	Dutch Organization for Applied Scientific Research
U.S. EPA	U.S. Environmental Protection Agency
U.S. FDA	U.S. Food and Drug Administration
WHO	World Health Organization

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Table 1: U.S. EPA's work on chemical mixtures risk assessment since 1986.

INTRODUCTION

Evaluation of potential health hazards from exposure to chemical mixtures or combined exposure to multiple chemicals in the food and environment poses one of the major challenges for toxicological research and risk assessment. Current risk assessment methods are largely based on evaluation of high dose toxicity data of individual chemicals. However, in most real world situations, exposures occur to a complex range of chemicals at low doses rather than to single chemical at a very high dose. An important factor in risk assessment of chemical mixtures is the availability of reliable data on the identity, levels of exposure, toxicokinetics, toxicodynamics and toxicological interactions for the whole mixture or its individual components. Regulatory agencies, scientific community and independent organizations worldwide (U.S. Environmental Protection Agency (U.S. EPA), U.S. Food and Drug Administration (U.S. FDA), Agency for Toxic Substances and Disease Registry (ATSDR), National Institute for Occupational Safety and Health (NIOSH), National Institute of Environmental Health Science (NIEHS), Flavor and Extract Manufacturers Association (FEMA), European Food Safety Authority (EFSA), Scientific Community on Health and Environmental Risk (SCHER), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), Scientific Committee on Consumer Safety (SCCS), Dutch Organization for Applied Scientific Research (TNO), Chemical Inspection and Regulation Service (CIRS), World Health Organization (WHO), Joint Expert Committee on Food Additives (JECFA), Organization for Economic Co-operation and Development (OECD), International Life Sciences Institute- Health and Environmental Sciences Institute (ILSI-

HESI)) have been conducting research and developing risk assessment tools for safety evaluation of chemical mixtures.

The ILSI North America Technical Committee on Food and Chemical Safety, 2013 Summer Fellowship Program focused on toxicology and risk assessment of chemical mixtures especially those related to food. The purpose of this project is to research the state of science of the toxicology and risk assessment methodologies for chemical mixtures and gather perspectives from regulatory agencies, scientific community and independent organizations regarding the on-going research on safety evaluation of chemical mixtures. The objectives of this project include:

- Extensive scientific literature search to summarize the list of tools/methods used for risk assessment of chemical mixtures worldwide, understand the differences in the risk assessment frameworks used by different agencies and the level of regulatory acceptance of these frameworks.
- Identify the most suitable risk assessment framework that could be applied to safety evaluation of chemical mixtures especially in food.
- Select criteria such as exposure levels, availability of data, toxicological interactions, route of exposure etc. for chemical mixtures risk assessment framework and conduct case studies using the most acceptable/suitable risk assessment framework.
- Future directions:
 - Identify the major data gaps and sources of uncertainties.
 - Studies that need to be conducted for better understanding of toxicology of chemical mixtures especially those in food.

- How to improve the current risk assessment practices for evaluating the effects of chemical mixtures on human health.

The very first objective of this project was to research the scientific literature and compile the work done by regulatory agencies worldwide in the field of risk assessment of chemical mixtures. Table 1 summarizes the frameworks and methodologies used by U.S. EPA since 1986 for risk assessment of chemical mixtures. It also includes some of the pesticides mixtures and polyaromatic hydrocarbon (PAH) mixtures case studies done by U.S. EPA over the past few years. The National Academy of Sciences established the paradigm for human health risk assessments and it includes a list of interconnected processes for performing a risk assessment -hazard identification, dose-response assessment, exposure assessment, and risk characterization (NRC, 1983). This paradigm is the foundation for U.S. EPA's work on chemical mixture risk assessment (U. S. EPA, 2000). The availability and quality of data determines the selection of a risk assessment method (U.S. EPA, 2000). There are two approaches- whole mixture approach and component based approach for risk assessment of mixtures. Whole mixture approach is taken when data is available on the mixture of concern or sufficiently similar mixture or groups of similar mixtures. If there is data available on the interactions of mixture components or whether the components act with a similar mode of action or are toxicologically independent, then component based approach of risk evaluation is followed.

Report	Agency	Scope	Approach/Methodologies	Assumption (s)
Guidelines for the Health Risk Assessment of Chemical Mixtures 1986 (U.S. EPA, 1986)	U.S. EPA	Procedural guide that emphasizes broad underlying principles of the various science disciplines (toxicology, pharmacology, statistics) necessary for assessing health risk from chemical mixture exposure.	<ul style="list-style-type: none"> • If toxicity data on the mixture of concern are available, the quantitative risk assessment is done directly from these preferred data. • When toxicity data are not available for the mixture of concern, toxicity data on a “sufficiently similar” mixture is used. • Evaluate the mixture through an analysis of its components, e.g., using dose addition for similarly acting chemicals and response addition for independently acting chemicals. • Incorporation of interactions data when available, if not as part of the quantitative process, then as a qualitative evaluation of the risk. 	<ul style="list-style-type: none"> • Interaction effects at low dose levels either do not occur at all or are small enough to be insignificant to the risk estimate. <p>Disadvantage- No single approach is recommended in this guidance document.</p>
Multi-pathways Exposures for Combustor Emissions 1998 (U.S. EPA, 1998a)	U.S. EPA	<ul style="list-style-type: none"> • Addresses Clean Air Act requirements. • Health risks of multi-pathway exposures to combustor emissions. 	<ul style="list-style-type: none"> • Exposures from different pathways. • No-observed-adverse-effect- level (NOAEL) to derive Reference Dose (RfD) or Reference Concentration (RfC). 	
Guidance for identifying pesticide chemicals and other substances that have a common mechanism of toxicity 1999 (U.S. EPA, 1999)	U.S. EPA	Studies the potential for a cumulative toxic effect and the magnitude of the effect in individuals exposed to pesticides and other substances that cause a common toxic effect by a common mechanism.	<ul style="list-style-type: none"> • Identify those pesticides and other substances that cause common toxic effects by common mechanisms. • Group them in accordance with commonality of toxic effect and toxic mechanism. • Combined risk assessments can be performed and the potential for cumulative toxicity that may result from exposure to substances within a group can be characterized. 	Possibility that low-level exposures to multiple substances that cause a common toxic effect by a common mechanism could lead to the same adverse health effect as would a higher level of exposure to any of the chemicals individually.
Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures 2000 (U.S. EPA, 2000)	U.S. EPA	<ul style="list-style-type: none"> • Update on 1986 guidelines for multiple chemicals to enhance methods. • Health risks for whole mixtures, for combinations of similar, independent, & interacting chemical. • Specific approaches to address complex mixture risk values, environmental transformations of complex mixtures, toxicological similarity based on varying evidence (from similar toxic mechanisms to similar target organs) and toxicological interactions. 	<p>Whole mixture</p> <ul style="list-style-type: none"> - Mixture of concern- Mixture RfD/C; slope factor. - Sufficiently similar mixture- Mixture RfD/C; slope factor. - Groups of similar mixtures- Comparative Potency. <p>Components</p> <ul style="list-style-type: none"> - Toxicologically similar- Hazard index (HI); relative potency factors (RPFs). - Toxicologically independent- Response addition. - Interactions- Interactions Hazard Index (HI). 	Not addressed approaches for assessing multi-pathway exposures to chemical mixtures as well as approaches for multiple effects from chemical mixture exposures.
National air toxics assessment (NATA) (U.S. EPA, 1998b)	U.S. EPA	<ul style="list-style-type: none"> • Inhalation health risks of outdoor air toxics from multiple sources. • Define baseline & driving chemicals/sources and prioritize data collection. 	<ul style="list-style-type: none"> • Cancer risks. • Non-cancer Chronic Reference Concentration. 	

Guidance on Cumulative Risk Assessment of Pesticide Chemicals That Have a Common Mechanism of Toxicity 2002 (U.S. EPA, 2002)	U.S. EPA	Studies potential human risks associated with multi-chemical and multi-pathway exposures to pesticides.	<ul style="list-style-type: none"> • Identification of a group of chemicals, a common mechanism group (CMG) that induces a common toxic effect by a common mechanism of toxicity. • Evaluate registered and proposed uses for each CMG member in order to identify potential exposure pathways (i.e., food, drinking water, residential) and routes (i.e., oral, inhalation, dermal). • Hazard characterization phase, the various endpoints associated with the common mechanism of toxicity are identified, as well as the test species/sex that might serve as a uniform basis for determining relative potencies among the chemicals of interest. • Exposures from minor pathways should be considered qualitatively. Thus, a subset of common mechanism chemicals to be included in the quantification of cumulative risk needs to be identified from the CMG. This subgroup is called the cumulative assessment group (CAG). • The guidance recommends use of dose addition for determining the combined risk of the CAG. • Relative Potency Factors (RFPs). 	Margins of exposure may be based on NOAELs for the common toxic effect rather than modeling dose-response curves of each chemical member to derive more refined relative potencies and points of departures.
Frame work of cumulative risk assessment 2003 (U.S. EPA, 2003)	U.S. EPA	Provides a conceptual framework for identifying the fundamental elements and basic principles to evaluate cumulative risks from a combination of chemical and nonchemical stressors.	<p>The framework is meant to include biologic, chemical, physical, radiologic, and psychosocial stressors – the focus is on real-world exposures rather than groups of agents with common characteristics.</p> <p>An overall analytical strategy is identified: Phase 1 – planning, scoping, and problem formulation. Phase 2 – analysis phase. Phase 3 – interpretation and risk characterization.</p> <p>Single stressor information- toxicological independence or similarity. Multiple stressor information- stressors interactions, joint chemical toxicity. Measures- decision indices, probabilistic or qualitative approaches, common metric, biomarkers.</p>	
Concepts, Methods and Data Sources for Cumulative Health Risk Assessment of Multiple Chemicals, Exposures and Effects: A Resource Document 2007 (U.S. EPA, 2007)	U.S. EPA	<ul style="list-style-type: none"> • Not a regulatory document and is not guidance but rather a presentation of concepts, methods and data sources. • Approaches for implementing cumulative risk assessments. • Focuses on two areas: initiating factors for a cumulative risk assessment with procedures for data collection and organization; and technical approaches for assessing and characterizing human health risks associated with a subset of cumulative risk issues (i.e., multiple chemicals, exposures and effects). 	<ul style="list-style-type: none"> • Identify population subgroups that are sensitive to the relevant chemicals or vulnerable to differential exposure. • Divide exposure/time groups further into subgroups in which chemicals are thought to cause toxicity by the same mode of action or affect the same target organ. • Factors to consider in forming these toxicity groups include toxicology of internal co-occurrence, pharmacokinetic parameters, persistence of the chemicals in the body and the formation of metabolites. • Use of Internal Doses in the Hazard Index. 	Grouping chemicals by the potential for co-occurrence and joint toxic action.

Table 1: U.S. EPA's work on chemical mixtures risk assessment since 1986.

CONCLUSION

This project is still in its initial stages and I am continuing to work on it. The next steps include more literature review of the work done by other agencies, comparing the different regulatory frameworks for safety evaluation of chemical mixtures and selecting the most appropriate framework for risk assessment of food related chemical mixtures.

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