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Role of functionalized liposomes in *drug delivery* through the blood-brain barrier

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ABSTRACT

The central nervous system (CNS), one of the most delicate microenvironments of the body, is protected by the blood-brain barrier (BBB). The protective properties of the BBB are conferred by the intricate architecture of its endothelium coupled with multiple specific transport systems expressed on the surface of endothelial cells in the brain vasculature. Although the BBB has a key role in regulation of neural biochemical environment, essential for maintaining neuronal integrity, it limits drug delivery to the CNS. In fact, less than 2% of all US Food and Drug Agency (FDA)-approved small-molecule drugs cross the intact BBB to varying degrees. Due to their flexible physicochemical and biophysical properties, the liposomes represent an attractive tool to deliver therapeutic molecules across the BBB. Moreover, their surface can be easily modified with ligands to improve their target and delivery. The peptide gH625, identified as a membrane-perturbing domain in glycoprotein H (gH) of *Herpes Simplex virus* 1, has been used extensively for vector-mediated strategies that enable passage of a large variety of small molecules as well as proteins across cell membranes *in vitro*. The goal of this Ph.D. project was to develop a new carrier system to deliver therapeutic molecules through the BBB with high efficacy and minimal toxicity. The project was divided in two parts. In the first part, *in vitro* and *in vivo* experiments were performed to investigate the capacity of gH625 peptide to enter and accumulate in neuron and astrocyte cell lines, and its ability to cross the blood-brain barrier in rats. In the second part of the project, the efficiency of liposomes functionalized with gH625 was evaluated on both *in vitro* model of rat BBB by using a neuroprotective peptide like PACAP (pituitary adenylate cyclase-activating polypeptide) and on *in vivo* mouse brain by using a hypothermic neuropeptide. The results show that gH625 peptide has a significant ability to penetrate brain cells. In fact, gH625 can be efficiently incorporated by human neuroblastoma (SH-SY5Y) and glioblastoma-astrocytoma cell lines (U-87 MG) without alteration of their cell viability. Furthermore, the *in vivo* experiments demonstrate that, despite the blood filtration action of the liver, carried out through the gH625 uptake of Kupffer cells, gH625 substantially reaches the brain BBB vessels. In particular, gH625 can be highly accumulated in endothelial cells of the BBB and taken up in some neurons. The results of second part of project show that the functionalization of liposomes with gH625 improves their passage through the endothelium of *in vitro* BBB model, thus resulting in an increased transport of PACAP and its less accumulation in the endothelial cells. Moreover, the toxicity studies reveal that the gH625-liposomes are nontoxic and do not affect tight junction organization in the BBB endothelium. Finally, further *in vivo* results demonstrated that the gH625 peptide may improve the efficiency of liposomes in mice. The results of this study suggest that gH625 peptide is a valuable tool to develop functionalized nanosystems for drug delivery to the brain. In particular, gH625-mediated liposomes represent a promising strategy to deliver therapeutic agents to CNS. Taken together, these data may have importance for the treatment of brain diseases and tracking of nanosystems *in vivo.*

Keywords: blood-brain barrier, drug delivery, peptide, liposomes, neurons, astrocytes

RIASSUNTO

Il sistema nervoso centrale (SNC), uno dei più delicati microambienti del corpo, è protetto dalla barriera emato-encefalica (BEE). Le proprietà protettive della BEE sono conferite dalla complessa architettura del suo endotelio associata ai sistemi di trasporto specifici espressi sulla superficie delle cellule endoteliali della vascolatura cerebrale. Anche se la BEE ha un ruolo chiave nella regolazione dell'ambiente biochimico neurale, essenziale per mantenere l'integrità neuronale, essa limita la somministrazione di farmaci per il SNC. Infatti, meno del 2% di tutte le piccole-molecole farmacologiche approvate dalla Food e Drug Agency statunitense (FDA) attraversano a vari livelli la BEE intatta. A causa delle loro flessibili proprietà fisico-chimiche e biofisiche, i liposomi rappresentano un interessante strumento per fornire molecole terapeutiche attraverso la BEE. Inoltre, la loro superficie può essere facilmente modificata con ligandi per migliorare la loro destinazione e consegna. Il peptide gH625, identificato come un dominio perturbante la membrana nella glicoproteina H (gH) dell'Herpes Simplex virus di tipo 1, è stato ampiamente utilizzato per strategie vettore-mediate che consentono il passaggio di una grande varietà di piccole molecole e proteine attraverso le membrane cellulari *in vitro*. L'obiettivo di questo progetto di dottorato è stato quello di sviluppare un nuovo sistema di trasporto per fornire molecole terapeutiche attraverso la BEE con elevata efficacia e tossicità minima. Il progetto è stato suddiviso in due parti. Nella prima parte dello studio, sono stati eseguiti esperimenti *in vitro* e *in vivo* per valutare la capacità del peptide gH625 di entrare e accumularsi in linee cellulari di neuroni ed astrociti, e la sua abilità di attraversare la barriera emato-encefalica in ratti. Nella seconda parte del progetto, l'efficienza di liposomi funzionalizzati con gH625 è stata valutata sia su un modello *in vitro* di BEE di ratto utilizzando un peptide neuroprotettivo come il PACAP (peptide attivante l'adenilato ciclasi pituitaria) sia *in vivo* sull'encefalo di topo utilizzando un neuropeptide ipotermico. I risultati dimostrano che il peptide gH625 ha una notevole capacità di penetrare le cellule cerebrali. Infatti, gH625 può essere efficientemente incorporato dalle linee cellulari di neuroblastoma (SH-SY5Y) e glioblastoma-astrocitoma (U-87 MG) umano, senza alterare la loro vitalità cellulare. Inoltre, gli esperimenti *in vivo* dimostrano che, nonostante l'azione di filtrazione del sangue del fegato, realizzata attraverso la captazione del gH625 da parte delle cellule del Kupffer, gH625 raggiunge sostanzialmente i vasi cerebrali della BEE. In particolare, gH625 può essere altamente accumulato nelle cellule endoteliali della BEE e internalizzato da alcuni neuroni. I risultati della seconda parte del progetto mostrano che la funzionalizzazione di liposomi con gH625 migliora il loro passaggio attraverso l'endotelio della BEE *in vitro*, risultando in un aumentato trasporto del PACAP e in un suo minore accumulo nelle cellule endoteliali. Inoltre, gli studi di tossicità rivelano che i gH625-liposomi non risultano tossici e non influenzano

l'organizzazione delle giunzione strette nell'endotelio della BEE. Inoltre, ulteriori risultati *in vivo* dimostrano che il peptide gH625 può migliorare l'efficienza di liposomi nei topi. In conclusione questo studio suggerisce che il peptide gH625 è un valido strumento per sviluppare nanosistemi funzionalizzati per la trasporto di farmaci al cervello. In particolare, i liposomi funzionalizzati con gH625 rappresentano una strategia promettente per fornire agenti terapeutici al SNC. Presi insieme, questi dati possono avere notevole importanza per il trattamento di malattie cerebrali e/o il monitoraggio di nanosistemi *in vivo*.

Parole chiave: barriera emato-encefalica, trasporto farmaci, peptide, liposomi, neuroni, astrociti

1. BLOOD BRAIN BARRIER (BBB)

The central nervous system (CNS) is the most sensitive and critical organ system in the human body. For optimal activity, the CNS requires a precise and balanced regulation of its microenvironment and homeostasis with conditions far different from those the rest of the organism. In the mammalian body, biological barriers at the interfaces between blood and neural tissue play essential role in this regulation (Abbott et al., 2006). Three barrier layers limit and regulate molecular exchanges at these interfaces: i) the blood-brain barrier (BBB) formed by the cerebrovascular endothelial cells between blood and brain interstitial fluid (ISF); ii) the choroid plexus epithelium between blood and ventricular cerebrospinal fluid (CSF); and iii) the arachnoid epithelium between blood and subarachnoid CSF (Abbott 2004). The BBB is probably the most selective and controlled of these barriers, playing critical roles in cognition, regulating metabolism and coordinating of peripheral organs functions. The presence of a hematoencephalic barrier was first demonstrated by Paul Ehrlich in 1885. He showed that in animals, after intravenous (iv) injection of trypan blue, the dye was stained peripheral tissues but not the brain. He concluded that the brain had a low affinity for the dye. In 1913, Edwin Goldmann demonstrated that after dye administration into the cerebrospinal fluid (CSF), the brain was stained but other tissues were not. Goldmann concluded that there was a barrier between the CSF and blood. Due to conflicting opinions, the existence of a BBB only has been completely accepted after the 1960 (Ribatti et al., 2006; Alyautdin et al., 2014)

The BBB ensures highly selective and efficient mechanisms to transport nutrients, regulate ion balance and prevent access of potentially toxic substances into the brain (Sanchez-Covarrubias et al., 2014). However, the BBB also significantly restricts the delivery of therapeutic molecules to the brain, preventing the effective treatment of many CNS disorders. Therefore, several strategies are currently investigated to enhance the delivery of drugs across the BBB. Moreover, a number of methods and models are available to evaluate the BBB permeation *in vivo* and *in vitro*, thus improving drug discovery for neurological diseases (Abbott et al., 2013).

1. 1 Morphofunctional aspects of the blood-brain barrier

The BBB is a critical interface between blood and neurons that acts as a physical and metabolic barrier. The main function of BBB is to establish and maintain homeostasis in the CNS (Bradbury 1993; Abbott and Friedman 2012). Due to its large surface area and the short diffusion distance between neurons and capillaries, the endothelium of BBB controls the influx and efflux of a wide variety of substances, including endogenous nutrients and exogenous compounds, by ensuring a homeostatic environment for the brain (Ballabh et al., 2004; Weiss et al., 2009). It guarantees to CNS essential nutrients and mediates efflux of many waste products. BBB, regulating fluid and ionic movements between the blood and the brain by specific ion

transporters and channels, produces an optimal medium for neuronal function know as the brain interstitial fluid (ISF) (Abbott NJ 2004). Moreover, the BBB protects the brain from changes in ionic composition which would disturb axonal and synaptic signaling (Cserr and Bundgaard 1984). BBB also keeps separated the pools of neuroactive agents and neurotransmitters that act in the CNS and in the peripheral tissues, so that similar agents can be used in the two systems without "crosstalk" (Abbott et al., 2006). Finally, BBB prevents entry of toxic substances into CNS, such as metabolites and neurotoxins (endogenous and xenobiotic), favoring the longevity of brain and avoiding premature cell death and neurodegeneration (Begley, 2004).

BBB in adult is formed of a complex cellular network, and the main components of this system are brain endothelial cells, pericytes, astrocytic end-feet and highly specialized basal membrane. These cellular components in concert with microglia, neurons and extracellular matrix are organized into well-structured neurovascular units (NVU) **(Figure 1)** (Chen et al., 2012). Disruption of any NVU component, due to physiological or pharmacological stressor, can alter BBB integrity, subsequently modifying brain microvascular permeability (Rolfe and Brown 1997; Oldendorf et al., 1977).

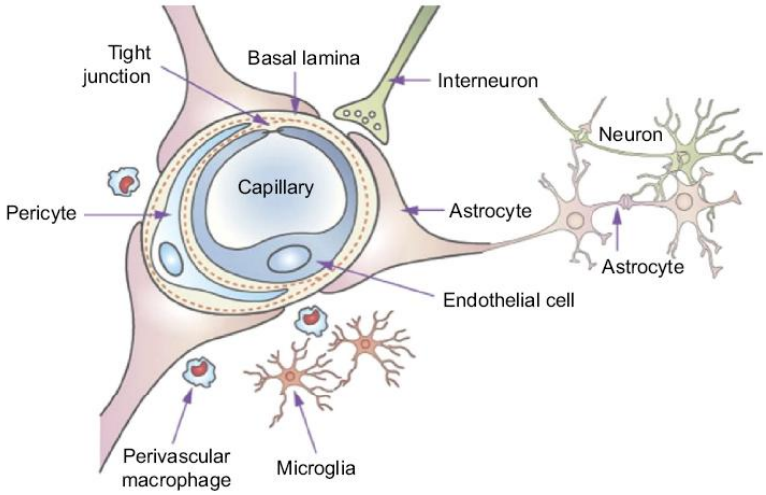

Figure 1: Cellular components of blood-brain barrier and neurovascular unit. Chen et al., 2012. *Adv Drug Deliv Rev, 64(7).*

Brain Endothelial Cells. BBB endothelial cells are also known as brain microvascular endothelial cells (BMECs). The BMECs, compared to peripheral vasculature, are characterized

by: absence of fenestrations, continuous tight junctions (TJs), increased mitochondrial content and minimal pinocytotic activity (Oldendorf et al., 1977; Takakura et al, 1991; Fenestermacher J et al., 1988). TJs reduce the paracellular permeability at the BBB (Hawkins and Davis, 2005; Zlokovic, 2008), increasing its transendothelial electrical resistance (TEER) (1500-2000 Ω cm^2) (Butt et al., 1990). BBB TJs consist of a complex interaction of three core protein components: transmembrane proteins (occludin, claudin and junction adhesion molecules), accessory proteins like membrane-associated guanylate kinase (MAGUK)-like proteins like zonula occludens (ZO-1, ZO-2, ZO-3, etc.) and cytoskeletal proteins (actin etc.) **(Figure 2)** (Bhowmik et al., 2015). Cell-cell interaction in the junctional zone is stabilized by adherens junctions (AJs) (Abbott NJ et al., 2006).

Occludin is a 60-65 kDa protein formed by four transmembrane domains: two extracellular loops, a long carboxy-terminal cytoplasmic domain and a short amino-terminal cytoplasmic domain. It is highly expressed at the BBB along cellular margins of the brain microvasculature (Lippoldt et al., 2000). Functional TJ-associated occludin assembles into dimers and oligomers via disulfide bond formation (McCaffrey et al., 2007). Several studies clearly demonstrated that the modulation of occludin oligomeric assemblies is associated with loss of BBB integrity and increased paracellular permeability (MCarthy et al., 1996) in pathological conditions (McCaffrey et al., 2008; Lochhead at al., 2010).

Claudin is a 20-24 kDa protein that has similar membrane topology to occludin, but not sequence homology (Furuse et al., 1998). At least 24 claudins have been identified in mammalian tissues, among these claudins-1, -3, -5, and -12 participate in the formation of tight junctions between BMECs (Liebner S et al., 2000a; Liebner S et al., 2000b; Nitta T et al., 2003; Schrade et al., 2012). The claudins form tight junctions through homophilic "claudin claudin" interactions mediated by their extracellular loops (Piontek et al., 2008). Together with occludins they can also assemble into heteropolymers to form intramembranous strands, containing fluctuating channels for selective diffusion of ions and hydrophilic molecules (Matter and Balda 2003). Each claudin regulates the diffusion of a group of molecules of specific size. Studies have shown that occludin localizes to the TJs only when claudins are already localized at the TJ. Thus, it is possible that claudins form the primary "seal" of the tight junctions (Ronaldson and Davis 2012; Kubota et al., 1999; Sanchez-Covarrubias et al., 2014).

Junction Adhesion Molecules (JAMs) are proteins formed by a single transmembrane domain and two immunoglobulin-like loops in the extracellular portion. They regulate the formation of tight junctions during the acquisition of cell polarity (Ebnet et al., 2004). In human, two JAMs are expressed in the tight junctions of BBB, JAM-A and JAM-C (Aurrand-Lions et al., 2001). JAM-A appears mediate early attachment of adjacent endothelial cells during BBB development through homophilic interactions and loss of JAMs is associated with BBB breakdown

(Ronaldson and Davis 2012; Dejana et al., 2000; Yeung et al., 2008; Hoffman 2009). Interestingly, JAMs are also implicated in the regulation of transendothelial migration of leukocytes (Dejana et al., 2000; Del Maschio et al., 1999).

Cytoplasmic accessory proteins are involved in tight junction formation. These include members of the membrane-associated guanylate kinase (MAGUK)-like proteins, like *zonula occludens* (ZO-1, ZO-2, ZO-3, etc.), cingulin, and several others. *Zonula occludens* proteins provide the cytoskeletal anchorage for the transmembrane tight junction and control spatial distribution of claudins (Hawkins and Davis 2005). The ZO proteins are directly associated with cytoplasmic domain of occludin (Rao 2009) and with carboxy-terminal of claudins (Itoh et al., 1999). ZO-1 is a 222 kDa phosphoprotein able to link TJ proteins, such as occludin, to the actin cytoskeleton, thus maintaining both function and stability of the TJs (Fanning et al., 1998). ZO-2 is a 160 kDa phosphoprotein, localized at the TJs and in non-TJ containing tissues (Hawkins and Davis 2005; Mark and Davis 2002), that may act as a "stand in" for ZO-1 under conditions in which ZO-1 and TJ protein interactions are disrupted (Sanchez-Covarrubias et al., 2014). ZO-3 is expressed in some TJ-containing tissues; however, its role at the BBB has not been elucidated (Inoko et al., 2003). Cingulins are actomyosin-associated proteins with large globular N-terminal "head" domain, coiled-coil "rod" domain, and small globular C-terminal "tail". Cingulin, by interacting with ZO proteins and junction adhesion molecules, helps the BBB formation (Bhowmik et al., 2015).

Adherens Junctions (AJs) are composed of multiple protein components including vascular endothelium (VE) cadherin, actinin and catenin (Vorbtodt and Dobrogowska, 2003). They are responsible for intercellular adherence between adjacent endothelial cells (Hawkins and Davis, 2005) by using homophilic interactions of VE-cadherin expressed on these cells. Cytoskeletal binding occurs via catenin accessory proteins. Specifically, β-catenin links VE-cadherin to α-catenin, an interaction that induces the direct binding to actin (Oldendorf et al., 1977; Sanchez del Pino et al., 1995). Disruption of protein-protein interactions within AJs can result in decreased BBB functional integrity (Ronaldson et al., 2009; Abbruscato and Davis 1999; Iyer et al., 2004).

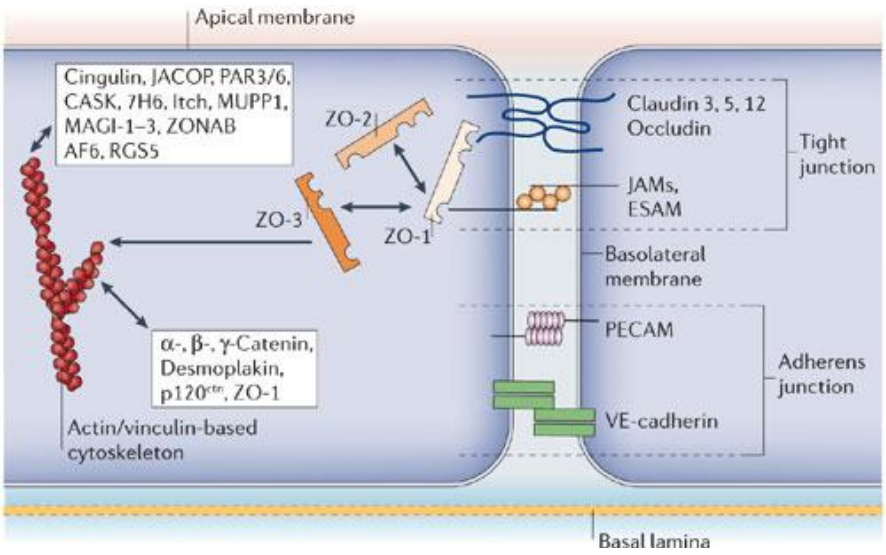

Figure 2: BBB tight junction. The tight junction (TJ) connects adjacent endothelial cells and forms the physical basis of the BBB. TJs components are: occludin, claudin and junction adhesion molecules (JAMs), accessory proteins (Zonulae occludins 1-3), cytoskeletal proteins and other molecules.
Abbott et al., 2006 *Nat. Rev. Neuro. 7: 41-53.*

***Pericytes*.** Pericytes are contractile cells attached to the abluminal side of brain capillary endothelial cells and on the luminal side of the astrocyte end-feet (Hawkins and Davis 2005; Saint-Pol et al., 2012). These cells have several functions: contribute to vascular contractility and immuno responses, help the formation of BBB (regulating the expression of BBB-specific genes in endothelial cells by inducing polarization of astrocytic end-feet surrounding CNS blood vessels) and also reduce the expression of molecules that increase vascular permeability (Armulik et al., 2010). The pericytes also play a role in maintenance of BBB TJ protein complex (Sa-Pereira I et al., 2012), by producing pericyte-derived angiopoietin, which induces occludin expression at the TJ in an *in vitro* endothelial-pericyte co-culture model (Hori S et al., 2004). Moreover, *in vitro* endothelial-pericyte co-culture studies have demonstrated that pericytes ensure proper localization of endogenous BBB proteins (i.e., P-gp, utrophin) in brain microvascular endothelial cells (Al Ahmad et al., 2011). Several studies *in vitro* and *in vivo* have shown that pericytes express many transporters including several multidrug resistance-associated protein (Mrp) isoforms (Berezowski et al., 2004), the P-glycoprotein (Pgp) (Bendayan et al., 2006), and cholesterol efflux regulatory protein (CERP) (Saint-Pol et al., 2012).

Astrocytes. Astrocytes are the most numerous cell types in the brain. They present a fibroblast-like morphology within grey matter (Raff et al., 1983). Several studies have shown that

astrocytes are fundamental for maintenance and induction of BBB characteristics. They support and protect neurons by controlling ion concentrations and neurotransmitter to maintain the homeostasis of CNS microenvironment, by modulating synaptic transmission and by regulating immune reactions (Rodríguez-Arellano et al., 2015; Keaney and Campbell 2015).They produce various inducing factors, including transforming growth factor beta (TGF-β), glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) that are involved in induction and regulation of BBB phenotype. Recently it has been demonstrated a heterogeneity of diffusion patterns around astrocytic end-feet (Nuriya et al., 2013). Some astrocytic end-feet can form tight networks to stop free diffusion of molecules across them (Mathiisen et al., 2010). Moreover, the astrocytes are able to prevent excitotoxicity induced by acute increases of glutamate in the brain, via expression of glutamate transporters EAAT1 and EAAT2 that are responsible for glutamate uptake into the astrocyte cell (Yi and Hazell 2006). Many other transporters and enzymes are expressed on astrocytes including P-gp, BCRP, and MRP isoforms (Pardrige et al., 1997; van Vliet et al., 2005; Aronica et al., 2005). The presence of efflux transporters in astrocytes suggests that they may act limiting drug permeation into the brain parenchyma or concentrate drugs within the brain extracellular fluid (Ronaldson and Davis 2012; Ronaldson et al., 2008).

Basal membrane. Endothelial cells and pericytes are embedded into the basal membrane and surrounded by astrocytic end-feet. This membrane consists mainly of laminin, proteoglycans, collagen type IV, heparan sulfate, fibronectin and other extracellular matrix protein (Farkas and Luiten 2001). The basal membrane has a direct impact on the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptor (Hynes 1992) and the regulation of endothelial tight junction protein expression by matrix proteins (Savettieri et al., 2000; Tilling et al., 1998). The potential function of this membrane is to restrict the movement of the solutes (Ronaldson and Davis 2012; Abbott et al., 2010). As consequence, the disruption of this extracellular matrix is strongly associated with increased BBB permeability in pathological conditions (Rascherb et al., 2002; Rosenberg et al., 1993). The extracellular matrix of the basal lamina acts as an anchor for the cerebral microvascular endothelium. This anchoring function is mediated via interactions between endothelial integrin receptors, lamin, and other matrix proteins. Moreover, the matrix proteins influence the expression of TJ proteins, thus suggesting its implication in maintaining TJ protein integrity (Hawkins and Davis TP 2005; Ronaldson and Davis, 2012).

Microglia. Microglia are cells derived from haematopoietic precursors that act as primary immune cells of the brain and are activated in response to systemic inflammation, trauma, and several CNS disorders (Kofler and Wiley 2011; Kettenmann et al., 2011). In “resting condition” they have a ramified morphology, characterized by a small soma and fine cellular processes. In conditions of pathophysiological stress the microglia can trigger changes cell morphology,

reducing complexity of cellular processes and switching from a ramified morphology to an amoeboid appearance (Kettenmann et al., 2011). The activation of microglia produces high levels of neurotoxic and proinflammatory mediators such as peroxide, TNF-α, nitric oxide, and proteases, all of which result in cell injury and neuronal death (Ronaldson and Davis, 2012). Moreover, the microglia activated alters TJ protein expression and increased BBB permeability (Huber et al., 2006). Microglial cells express also transporter proteins, including P-gp, BCRP, MRP-4, and MRP-5, and the inflammatory events may change the mRNA/protein expression of these transporters (Berezowski et al., 2004). The presence of these transporters indicates a possible implication of microglia in CNS drugs permeability and distribution. However, until now little is known about the role of these cells in drug uptake into the CNS, more studies are required to elucidatetheir role.

Neurons. The cerebral microcirculation respond to metabolic requirements of CNS tissue via direct innervation of brain microvessel endothelial cells and associated astrocyte processes with noradrenergic (Cohen et al., 1997), serotonergic (Cohen et al., 1996), cholinergic (Tong and Hamel, 1999; Vaucher and Hamel, 1995) and GABAergic (Vaucher et al., 2000) neurons. It has been demonstrated that loss of direct noradrenergic input from the *locus coeruleus* is responsable of increased BBB susceptibility to effects of acute hypertension, resulting in significantly increased permeability to ^{125}I-labeled albumin (Berezowski et al., 2004). Moreover, stimulation of the parasympathetic sphenopalatine ganglion is able to induce BBB opening resulting in an increase in delivery of chemotherapeutic agents to the brain (Yarnitsky et al., 2004). Several studies showed that many factors that regulate neuronal growth, development, and repair also modulate endothelial cell function. As for VEGF, this factor supports neuronal growth and promotes neuronal migration in the developing CNS (Rosenstein et al., 2010) but is also upregulated under hypoxia conditions and this involves in increased BBB permeability (Davis et al., 2010). These data support the conclusion that the communication between neurons and endothelial cells not only regulate the blood flow in function of metabolic requirements of nervous tissue but also BBB permeability.

Finally, it should be noted that not all CNS is protected by the BBB. The absence of BBB in some areas determines the free passage of several molecules, due to the fenestrations in the endothelium of their brain capillaries. Among these areas there are circumventricular organs (CVOs) and pineal gland. The main function of the CVOs is control chemical composition of the cerebrospinal fluid (CSF) and release of hormones and neurotransmitters. The CSF provides to drain molecules or products of metabolism that are diluted and subsequently removed (Sanchez-Covarrubias et al., 2014).

Overall, the area with fenestrated capillaries is much less than the total area of the BBB, making negligible the high permeability of these areas compared to the ability of the endothelium of brain act as a barrier (Begley 1996; Mimee et al., 2013; Ufnal et al., 2014).

1.2 Transports across the blood-brain barrier

The BBB endothelium is the largest interface between the vasculature and the nervous system (12-20 m^2 for human brain). Due to short diffusion distance to neurons, the BBB endothelium represents the principal site to exchange molecules between the blood and the CNS (Abbott 2013).

Small lipophilic molecules (molecular weight less than 400 Da) and gaseous molecules such as O_2 and CO_2 can cross the BBB by passive diffusion (Biddlestone-Thorpe et al., 2012; Abbott et al., 1996). These molecules move across endothelium BBB membranes without energy expenditure or carrier protein, by following their concentration gradient. In general, the ability of a substance to pass for passive diffusion depends by its molecular size, concentration in blood, polarity, lipid solubility, and surface area available for diffusion. Small and lipid soluble substances can more easily pass across the BBB than large, polar and hydrophilic substance. However, the size limitations can be overcome by lipid solubility, especially for molecules highly hydrophobic (Habgood et al., 2000). The passive diffusion is also influenced by hydrogen bonding capability of a compound; if the number of hydrogen bonds formed by a compound is low, greater is its ability to passively diffuse across a membrane (Wolka et al., 2005). Generally, small, polar, or charged molecules, even if there are some limitations due to their physicochemical properties, can traverse the BBB using aqueous channels. There are some drugs that cross the BBB by passive diffusion such as steroids and opioids (morphine, diphenhydramine and heroin) (Wolka et al., 2005; De Gregori et al., 2012; Au-Yeung et al., 2006).

Nutrients, ions, and other molecules essential for CNS metabolism can cross the BBB by several specific transport mechanisms embedded in the apical and basal membranes (Ohtsuki and Terasaki 2007). The endogenous transport systems present on the BBB are following: i) Carrier-Mediated Transport (CMT) that consists in interactions of a substrate with carrier protein; ii) Endocytosis that involves vesicular transport via receptor-mediated, adsorptive, or bulk-phase endocytosis (Abbott et al., 2006; Wolka et al., 2005; Simionescu et al:, 2002); iii) Active transport, an energy dependent transport usually coupled to ATP-hydrolysis (Abbott et al., 2006; Abbott and Romero 1996) **(Figure 3)**.

Moreover, the presence of efflux transporters in the apical membrane restricts access of many potentially toxic components into the brain (Abbott 2013).

Leukocyte transport across the BBB is also highly regulated. Under healthy conditions there is a low expression of luminal adhesion molecules, with which the leukocytes interact (Engelhardt and Ransohoff 2012), therefore studies on leukocytes transport derived by disease model (Zlokovic 2008).

In addition, several enzymes such as monoamine oxidase, peptidase and nucleosidase are present on the BBB, suggesting that this structure is not only a physical and biological barrier but also a metabolic barrier. Alkaline phosphatase is present on the apical and basal membranes

of BBB endothelial (Bendayan et al., 2002); cytochrome P450 HE1 is expressed not only in the brain microvessels but also in the end-feet of astrocytes, whereas cytochrome P450 P450 HB1/2 is detected in endothelial cells and pericytes (Decleves et al., 2011). Several other enzymes play an important role in the degradation and metabolism of various substrates, including drugs, by adjusting their concentrations in the brain.

However, the highly controlled routes across the BBB are influenced by the health status of the individual, by systemic pathologies and by pathologies with specific CNS involvement (Abbott et al., 2010; Abbott and Friedman 2012; Muldoon et al 2013). Therefore, to develop therapeutic delivery strategies to CNS it is necessary to consider not only the possible routes for transendothelial movement of molecules but also the modifications of these routes in diseases conditions.

Carrier-mediated transport

The carrier-mediated transport (CMT) involves the transport of polar substances using proteins/carriers present in luminal and abluminal membrane. These proteins can be bidirectional vectors, providing to diffusion of substances in direction of concentration gradient; unidirectional vectors promoting the transport in only one direction (in or out the cells); or exchangers/co-transporter using exchange or co-transport of other solutes or ions in the same direction or in opposite direction (Begley et al., 2008). In particular, CMT helps the transport of nutrients such as esosis (glucose and galactose), amino acids, monocarboxylic acid (pyruvate, lactate, ketone bodies), nucleosides, purine (adenine and guanine), ammine (choline) and vitamins (Hawkins et al., 2006; Simpson et al., 2007, Ohtsuki and Terasaki 2007; Deeken and Loscher 2007; Spector and Johanson 2007). Generally, these nutrients are transported from blood compartment to brain, depending on CNS metabolic necessity and nutrients concentration in the blood (Zlokovic 2008).

Glucose uses a system of transport carrier mediated. The carrier for glucose transport is a member of glucose transporters family (GLUTs). GLUT1, -3, -4, -5, -6, and -8 are expressed in the brain. However, for the low affinity for glucose of GLUT5 and -6, and the very restricted expression of GLUT4, -6, and -8, probably GLUT1 and -3 are the most effective transporters of glucose in the CNS (Alyautdin et al., 2014). GLUT 1 is localized to luminal and abluminal membrane of the BBB and its transport is concentration-dependent (Yoshizumi et al., 2012; Samad et al., 2007). Two GLUT1 isoforms (45 and 55 kDa) have been identified, different only for the extent of glycosylation but not for structure or kinetic parameters (Birnbaum et al., 1986; Simpson et al., 2007).

The syndrome of GLUT1 deficiency in endotheliocytes involves a reduction of glucose levels in CSF which determine hypotension, growth retardation, convulsions, microcephaly, and movement disorders (ataxia and spasticity) (Vorbrodt et al., 2001; Klepper and Voit, 2002). GLUT3 is expressed in several tissues with very high glucose metabolism and its expression

increases in neurons during nervous system development. Moreover, also cationic, anionic, and neutral amino acids use the CTM. Three Na^+-dependent transporters for excitatory amino acids and one transport system for glutamine are located on the abluminal surface of ECs. The transport systems to deliver glutamate and glutamine are expressed on the luminal surface of ECs. This kind of organization promotes the entry of glutamate into the brain and removal of acidic and nitrogen-rich amino acids from the brain (Clark 2003). Transporters of neutral amino acids (NAAs) are expressed on both ECs membranes, to transport essential amino acids to the brain, and four Na^+-dependent NAA transporters located on the abluminal membrane preserve the concentration of these amino acids (Vorbrodt et al., 2001). Drugs delivery to CNS using CMT has been demonstrated. The Na^+-independent large neutral amino acid transporter (LAT-1) can transport of L-Dopa to brain for treatment of Parkinson's disease (del Amo et al., 2008).

Endocytosis

Macromolecules transport across the BBB occurs to using endocytic mechanisms. Vesicular transport comprises: receptor-mediated transport, adsorptive transport, and bulk-phase endocytosis (Abbott et al., 2006; Wolka et al., 2003; Simionescu et al., 2002).

In receptor-mediated transcytosis (RMT) the macromolecule interacts with specific receptors expressed on the surface of ECs that leads to endocytosis of substrate-receptor complex. The internalization of this complex into the intracellular compartment occurs by invagination of the luminal membrane and by encapsulation of complex in vesicles. Subsequently substrate dissociation from the receptor occurs and the vesicles release their contents in the intracellular compartment or their contents can be released directly into brain parenchyma by fusion with the abluminal membrane (Pardridge 2012).

The transport of proteins, such as transferrin (Jefferies WA et al., 1984; Chen and Liu 2012; Xiao and Gan 2013), the low-density lipoprotein (LDL) (Meresse et al., 1989), leptin (Zlokovic et al., 2000; Hsuchou H et al., 2011), immunoglobulin G (Deane et al., 2005; Xiao and Gan 2013), insulin and insulin-like growth factors (IGF-I and IGF -II) (Pardridge 2005) is mediated by specific transporters. Several other macromolecules are transported to CNS by RTM such as apolipoprotein E (ApoE), lactoferrin, epidermal growth factor (EGF), glutathione and diphtheria toxin are among these (Abbott et al., 2010; Rip et al., 2009). RMT occurs also for neuropeptides, such as arginine-vasopressin (AVP) (Zlokovic et al., 1990), inhibitory factor-1 tyrosine melanocyte-stimulating (Tyr-MIF-1), release luteinizing hormone-releasing (LHRH), some cytokines and chemokines (Banks 2006). Transport systems of the peptide-1 and peptide-2 (PTS-1 and PTS-2) mediate efflux of enkephalins, Tyr-MIF1 and AVP, respectively, from the brain to the blood (Banks 2006). PTS-3, on the luminal membrane, carries the T peptide in the brain; while PTS-4 allows the two ways shipping of LHRH.

The adsorptive transcytosis (AET) is an unspecific endocytic process governed by interactions of positively charged macromolecules with the negatively charged cell surface and subsequent

transcytosis and exocytosis (Sauer et al., 2005). The anionic sites are due to presence of acidic glycoproteins on the luminal membrane (Scherrmann 2002; Tamai and Tsuji 2000). Macromolecules such as heparin, cationic proteins, cell-penetrating peptides (Tat-derived peptides, penetrain, Syn-B vectors and transportan) enter in CNS by AET (Chen and Liu 2012).
Another transport mechanism is known as bulk-phase endocytosis. This transport involves uptake of substances solubilized in extracellular fluid without using of receptor (Sanchez-Covarrubias et al., 2014). This transport is characterized by formation clathrin cages on the cytoplasmic surface of the cell membrane, followed by invagination of the membrane and subsequent generation of a closed vesicle. The vesicle detaches from the membrane via membrane fission and, like for RMT, the releases its contents in the intracellular space or can fuse to with the abluminal membrane (Boron and Boulep 2005; Sanchez-Covarrubias et al., 2014).

Active transport

The delivery of some essential nutrients, ions and other endogenous substances into the CNS occur by an energy dependent transport, usually coupled to ATP-hydrolysis (Abbott et al., 2006; Abbott and Romero 1996). Other active transport mechanisms are responsible also to block/regulate access of potentially toxic molecules (Abbott et al., 2006). However, these kinds of transports allow the passage of substances against their concentration gradient.
Several therapeutic molecules reach the CNS by active transport processes, such as opioid analgesic peptides, opioid analgesic drugs, HIV-1 protease inhibitors, HMG CoA reductase inhibitors, antineoplastic agents, calcium channel blockers, cardiac glycosides and antibiotics (Sanchez-Covarrubias et al., 2014).

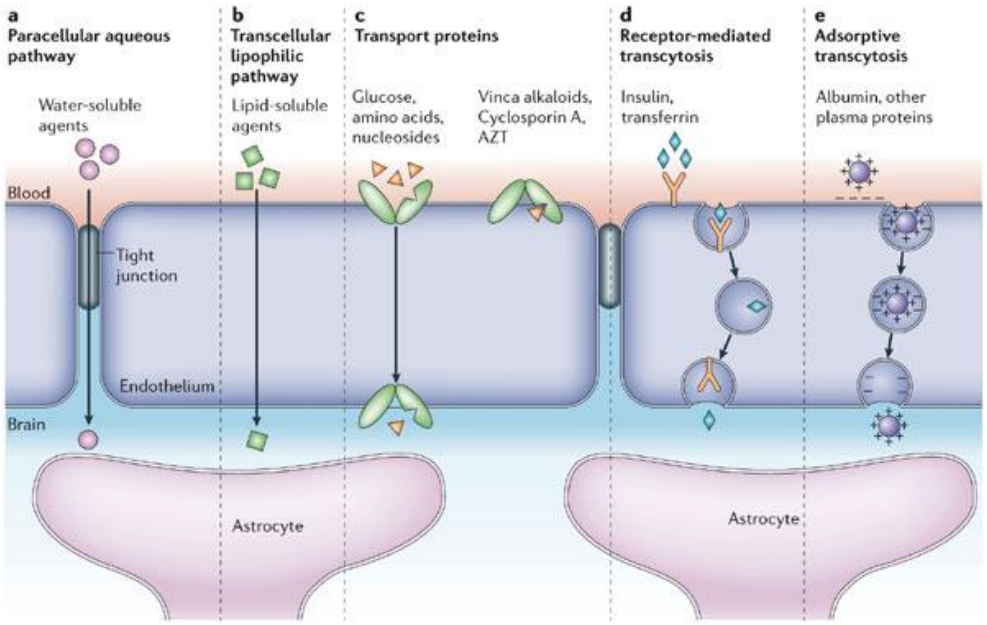

Figure 3: Transport across the BBB. Representation of several transport mechanisms on brain endothelial cells. **a)** paracellular aqueous pathway across tight junctions; **b)** transcellular pathways including the lipophilic pathway; **c)** transport proteins (carriers); **d)** receptor-mediated transcytosis; and **e)** adsorptive transcytosis. Abbott et al., 2006. *Nat. Rev. Neuro. 7: 41–53*

1.2.1 Transporters of BBB

Uptake and extrusion of various compounds and metabolites into the brain is mediated by transport proteins. Many influx and efflux transporter systems have been identified on the BBB including ATP-binding cassette (ABC) transporters, solute carrier (SLC) transporters and peptide transporters.

ABC Transporters

ABC transporters are drug efflux pumps that require ATP hydrolysis as a source of biological energy to transport substances against a concentration gradient in a single direction across membranes (Dean et al., 2001; You 2007). There are 48 genes encoding ABC transporter superfamily of proteins, which are subdivided into 7 distinct subfamilies (ABCA to ABCG) (Moitra 2012). All ABC transporters have three highly conserved motifs: Walker A, Walker B motifs and a motif involved in substrate recognition and ATP hydrolysis know as ABC signature C motif (i.e., ALSGGQ) (Sharom 2008; Hollenstein et al., 2007).These proteins are localized at the luminal side of brain capillaries of BBB and the prevent brain uptake of a large variety of lipophilic molecules, potentially toxic metabolites, xenobiotics. Moreover, they limit

the drugs entry into the brain reducing the efficacy of treatment of neurological disease (You 2007; Ronaldson 2007). The most studied members of the ABC superfamily in CNS are P-glycoprotein, breast cancer resistance protein, and members of the multidrug resistance related proteins (Begley DJ 2004).

a) P-glycoprotein (P-gp) is a 170-kDa efflux transporter encoded by multidrug resistant (MDR) genes (Gottesman et al., 1995) present to the luminal and abluminal sides of BBB endothelium (Bendayan et al., 2006) and detected also in astrocytes, microglia and neurons (Golden and Pardridge, 1999; Schlachetzki and Pardridge, 2003; Ronaldson et al., 2004; Lee et al., 2001; Volk et al., 2004).Two MDR isoforms have been identified in human tissues, MDR-1 and MDR-2 (Chen et al., 1986; Roninson et al., 1986). The MDR1 gene product is 1280 amino acids in length and has two homologous halves; each consists of six transmembrane domains and ATP- binding site. On the first extracellular there are loop two to four glycosylation sites are present (Loo and Clarke, 1995).

P-gp protects nervous tissue from exposure to potentially neurotoxic xenobiotics and maintains the precise CNS homeostatic environment (Sharom, 2007). It binds generally non-polar, weakly amphipathic compounds with different molecular size including cytokines, steroid hormones, peptides and lipids (Litman et al., 1997). Moreover, P-gp extruders from brain tissue an high number therapeutic substrates such as antibiotics, chemotherapeutics, immunosuppressants, anti-epileptics, anti-depressants and HIV-1 protease inhibitors (Sun et al., 2004; Demeule et al., 2002). The use of inhibitors of P-gp to increase the delivery of drugs to the brain has been evaluated, but the ubiquitous presence of P-gp in the rest of the body combined with the high dose of inhibitors has induced systemic toxicity (Thomas and Coley 2003).

b) Breast Cancer Resistance Protein (BCRP) is a transporter of 655 amino acids and with molecular weight around 72 kDa, identified in the MCF-7/AdrVp breast cancer cell line (Chen et al., 1990). It is composed of six transmembrane domains with the C- and N-termini located on the intracellular side of the plasma membrane (Doyle et al., 1998) with two to three sites for N-linked glycosylation in extracellular loops. It has demonstrated that these glycosylation sites do not influence the functional capabilities of the transporter or its cellular localization (Diop and Hrycyna 2005; Mohrmann et al., 2005). To maintain the efflux activity, BCRP forms functional homo- or heterodimers (Nakanishi et al., 2003; Graf et al., 2002, 2003). BCRP is expressed at the luminal side of BBB capillary endothelial cells, in astrocytes and in microglia (Eisenblatter et al., 2003; Zhang et al., 2003; Lee et al., 2007).

BCRP can transport physiological substrates (folic acid, glutathione and steroid hormones) (Mao and Unadkat 2005), and therapeutic compounds such as chemotherapeutic agents (Chen et al., 1990; Nakagawa et al., 1992; Keller et al., 1997; Rabindran et al., 1998; Allen et al., 1999).

In particular, there is significant overlap between substrates specificity of BCRP and substrates specificity of P-gp (Staud and Pavek 2005).

c) Multidrug Resistance Proteins (MRPs) family has 9 homologues, designated as MRP1-9 isoforms, with overlapped substrate profiles. Expression of MRP1-6 has been observed in human brain (Nies et al., 2004), whereas multiple MRPs like MRP1, 4, and 5 have been detected in the human BBB (Zhang et al., 2003; Bronger et al., 2005; Miller et al., 2000). Structural similarity has observed in MRP1, 2, 3, and 6, as each of them possesses three transmembrane domains (TMD) designated as TMD0, TMD1, and TMD2, respectively. TMD1 and TMD2 contain six alpha helices, whereas TMD0 contains only 5 alpha helices (Borst et al., 2000; Dallas et al., 2006). Seems that TMDs are assembled in the plasma membrane pore through which the transport of substrates occurs (Borst et al., 2000). MRP4 and MRP5 have structural similarity with P-gp that lack TMD0 (Borst et al., 2000; Lee 2004) but in all MRP homologues, the conserved cytoplasmic linker (L0) portion is essential for transport function. Nucleotide-binding domains are in the cytoplasmic region of the protein between TMD1 and TMD2 as well as between TMD2 and the C-terminus (Borst et al., 2000). The main function of MRPs is the extrusion of xenobiotics from cells, but their substrate profile is more restrictive from P-gp. MRPs isoforms generally transport organic anions and their glucuronidated, sulfated, and glutathione-conjugated metabolites (Zhang et al., 2000). Moreover, MRPs play a crucial role in drug efflux transport through the BBB. MRP1, 4 and 5 are present on the luminal membrane of brain ECs (Zhang et al., 2000; Dallas 2006).

MRP1 is able to transport several substrates such as glucuronide conjugates and sulfate conjugates (Sun et al., 2004), methotrexate (Borst et al., 2000), metalloids in the form of oxoanions, various toxins, carcinogens and drugs (Leslie et al., 2001). MRP2 is very similar to that of MRP1; however, many of its substrates show lower affinity for MRP2 than for MRP1 (Nies, 2007). MRP3 can transport antineoplastic drugs such as methotrexate and teniposide, but not vincristine, cisplatin, or doxorubicin (Borst et al., 2000; Dallas et al., 2006), and is also present on the plasma membranes of astrocytes and microglia (Bart et al., 2000). MRP4 transports a variety of compounds and is able to confer resistance to cells to HIV-1 therapeutics such as azidothymidine monophosphate (AZT-MP) and 9-(2phosphonylmethoxyethyladenine) (PMEA) (Lee et al., 2001). MRP5 has as characteristic the apparent intracellular expression of the protein and also a substrate unique, the anti-HIV-1 drug stavudine monophosphate (Kruh and Belinsky 2003). Finally, MRP6 interacts with cisplatin, etoposide and doxorubicin but not with methotrexate and vincristine (Dallas et al., 2006).

Solute Carrier (SLC) Transporters

Solute carrier superfamily transports anionic and cationic small molecules, nucleosides and peptides through plasma membrane. SLC superfamily comprises 43 subfamilies of SLC

transporters (SLC1-SLC43). SLC15A1, SLC16, SLC21, SLC22, SLC28, and SLC29 are present on the BBB (Kusuhara and Sugiyama 2005). SLC transporters do not require ATP to transport substrates across BBB but they use electrochemical or concentration gradients of solute. Therefore, these transporters are known as either facilitated transporters or secondary active transporters (Ronaldson and Davis 2012; You 2007). The major SLC transporters include proton coupled oligopeptide transporters, monocarboxylate transporters, organic anion polypeptide transporters, organic ion (anion and cationic) transporters, and nucleoside transporters (Hediger et al., 2004; Molina-Arcas et al., 2009).

- Proton coupled oligopeptide transporters (POT) are able to transport small peptides across the BBB by an electrochemical proton gradient (Carl et al., 2010). In addition to above-mentioned peptide transporters, there are also peptide transport system (PTS) expressed endogenously at the BBB endothelium. In the BBB, there are seven peptide transport systems (PTS1-PTS7). PTS2, PTS4, and PTS6, are bidirectional, the others are unidirectional (they facilitate either blood-to-brain or brain-to-blood peptide transport) (Banks and Kastin 1994). For example, analogs of PACAP (pituitary adenylate cyclase-activating polypeptide), a polypeptide with endocrine and vasodilatory properties, are transported through the BBB by PTS-6 (Banks et al., 1993; Dogrukol-Ak et al., 2009; Vaudry et al., 2000). In particular, a 38 amino acid PACAP analog is transported from blood to brain by a saturable carrier-mediated uptake component of PTS-6 (Banks et al., 1993), whereas CNS uptake of a 27 amino acid PACAP analog occurs by passive diffusion and a PTS-6 efflux component (Dogrukol-Ak et al., 2009). Some studies have been demonstrated that to enhance the brain delivery of peptide therapeutics and/or to preserve CNS concentrations of pharmacologically active peptides is possible to block the efflux component of PTS-6 (Banks et al., 1993; Dogrukol-Ak et al., 2009)

- Monocarboxylate transporters (MCTs) facilitate the rapid transport of monocarboxylates through the biological membranes. In brain, MCTs assist the transport of the monocarboxylates for uptake into the neurons and mediate the transport of some drugs across the BBB (Halestrap and Wilson 2012).

- Organic Anion Transporters Polypeptides (OATPs) are membrane influx transporters expressed in BBB to regulate cellular uptake of endogenous compounds and clinically important drugs (Niemi 2007). The OATP dependent transport is conducted by electrochemical gradients using. The OATPs family members OATP1A2, 1C1, 2A1, 2B1, 3A1, and 4A1 are present in human brain (Hagenbuch and Meier 2004).

- Organic Ion Transporters are the members of SLC transporter 22 superfamily (SLC22A) and can be classified into two specific types: organic anion transporters (OATs) and organic cation transporters (OCTs) (Kusuhara and Sugiyama 2004; Jonker and Schinkel 2004). The OATs determine movement of various endogenous molecules like anionic metabolites of neurotransmitters, prostaglandins, hormones, and exogenous molecules such as different drugs across the biological membrane (Riedmaier et al., 2012). In brain, OAT3 is the most highly expressed isoform and is present in the abluminal (brain side) and brush-border membrane (CSF side) of brain capillary endothelial cells and choroid plexus epithelial cells, respectively (Mori et al., 2003; Sweet et al., 2002). OCTs regulate cellular influx and efflux of various cationic substrates (Gründemann et al., 1997; Ciarimboli and Schlatter 2005).

- Nucleoside Transporters regulate the transport of nucleosides that is crucial for proper neuronal function (Lauder 1993). As brain cannot synthesize nucleosides *de novo*, the recycling pathways for nucleosides transportation is necessary. Depending on the Na^+ dependence nucleoside, the membrane transporters divided into two subcategories: concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) (Mackey et al., 1998; Gray et al., 2004).

1.3 *In vitro* BBB models

Several *in vitro* models of the BBB are available to study mechanisms to drugs transport to the brain, and biological and pathological processes related to the BBB (Cecchelli et al., 2007) **(Figure 4)**. The BBB *in vitro* models were established from various species, including rat, mouse, pig and bovine. These models derive from isolated brain capillary endothelial cells, used as primary cultures or as immortalized brain endothelial cell lines initially derived from primary cells. The BMECs can grown alone or in co-culture (with astrocytes or pericytes), and generate tight endothelial barriers with high transendothelial electrical resistance (TEER) (Molino et al., 2014). Generally, the BBB *in vitro* models preserve well the TJs and transport mechanisms identified *in vivo*, although there are some species differences (Abbott 2013). An ideal *in vitro* BBB model would replicate all aspects of the *in vivo* human brain endothelium. This is hard to realize because the cells are taken out of their normal brain microenvironment and grown in a simplified system/medium, lacking many of the influences from blood and brain side. Static and dynamic models are available, the latter involving flowing fluid on the endothelial luminal surface (Naik and Cucullo 2012). However, most permeability screening is still done on static systems, with cells grown on a porous filter in "transwell" configuration. The use of transwell offer a better quantification and resolution of permeation, minimizing the quantity of compound required (Wilhelm et al., 2011).

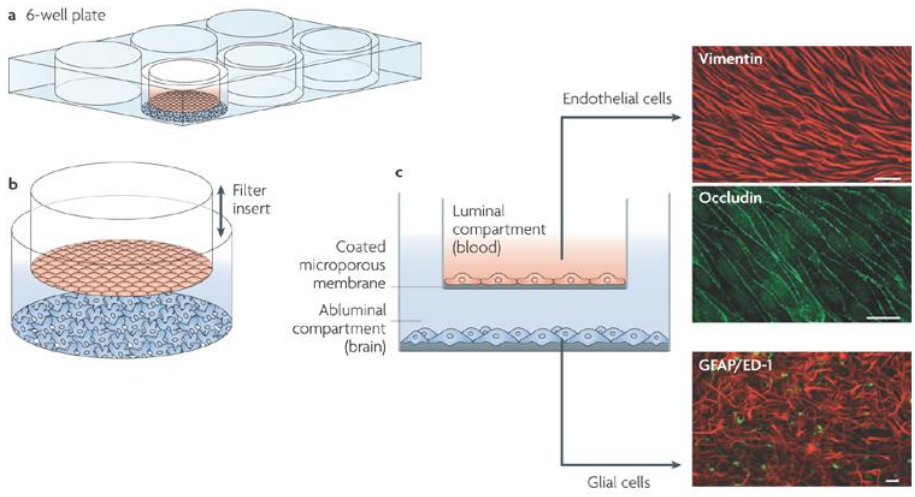

Figure 4: *In vitro* model of BBB. **a)** Brain endothelial cells are grown on transwell in co-culture with glial cells at the bottom of well culture plates; **b)** Glial soluble factors secreted in the culture medium induce the BBB phenotype in the capillary endothelium; **c)** Immunostaining for several endothelial cells and glial cells components (Cecchelli et al., 1999). Scale bar represents 25 μm.
Cecchelli et al., 2007 *Nature Reviews Drug Discovery 6, 650-661.*

It is necessary to use an *in vitro* BBB that reflects the *in vivo* condition, especially for expression of receptors and RTM pathways (Abbott 2013). Co-culture with astrocytes maintains the differentiated phenotype of receptors and transcytosis more similar to *in vivo* BBB conditions as demonstrated for LDL, transferrin, beta-amyloid, lactoferrin and IL-1 and others (references in Candela et al., 2010; Skinner et al., 2009). The inclusion of pericytes in cell culture models can be useful, depending on the differentiation state of the pericytes (Thanabalasundaram et al., 2011); they have effects on BBB induction and stabilization (Nakagawa et al., 2009; Vandenhaute et al., 2011). The hCMEC/D3 cell line, immortalized cell line, is widely used as human *in vitro* BBB model. However, hCMEC/D3 cells have some limitations, they are generally less tight than primary cultured cells and deficient in some TJs, enzymes and transport systems (Wilhelm et al., 2011). Moreover, complex 3-D cultures and growth in tubes with luminal flow have also been developed. However, tri-culture and 3-D models are complicated to set up and maintain, and they have not yet been fully assessed for RMT (Naik and Cucullo 2012). Recently, human *in vitro* BBB models from induced pluripotent stem cells (iPSCs) have been developed (Lippmann et al., 2012, 2014). These models are based on co-culture of iPSC derived ECs with astrocytes. Moreover, a human *in vitro* model, based on the co-culture of human endothelial cells derived from cord blood endothelial progenitor cells with astrocytes, shows for the first time a correlation with *in vivo* human data (Cecchelli et al., 2014). In conclusion, *in vitro* BBB model is a valuable tool for research community to study

BBB molecular mechanisms in physiological and pathological conditions and to test BBB permeability to therapeutic molecules, thus improving the development of new delivery strategies to CNS.

2. DRUG DELIVERY ACROSS THE BLOOD-BRAIN BARRIER

BBB not only plays a critical role in the brain protection against toxic and infectious agents, but also creates an obstacle for effective systemic drug delivery to the CNS. The high impermeability and selectivity of the BBB preclude the transport of many therapeutic molecules to the CNS thus making ineffective the treatment of several neurological diseases (Dominguez 2014). Moreover, many drugs for neurological disorders not achieve effective concentration within the brain tissue following intravenous or oral administration. Enhance dose or extend administration of drugs, to avoid this problem, often significantly increases systemic toxicity risk. During the last decade, drug delivery into the brain has been extensively investigated and several therapeutic strategies have been developed. Until now, these strategies are classified in two great categories: invasive and noninvasive techniques (Lu et al., 2014). However, despite numerous advances, pharmacological treatment of neurological disorders represents still a challenge for scientific community.

2.1 Invasive techniques

To deliver sufficient amounts of drugs to the CNS by systemic routes, invasive neurosurgery-based techniques are proposed. They provide BBB disruption or drugs administration directly into the brain tissue (Wang et al., 2002).

2.1.1 Intracerebral implants and intraventricular infusion

The most direct way to provide high drug concentrations into the brain, decreasing systemic exposure and toxicity, is to use intracerebral implants or intraventricular infusion (Scherrmann et al., 2002). Intracerebral implantation of therapeutic agents has been utilized to deliver into CNS several drugs such as antiepileptic drugs and some chemotherapeutics (Vukelja et al., 2007; Sheleg et al., 2002; DiMeco et al., 2002). In a rat epileptic model, the administration of phenytoin by implantation of polymers near the seizure focus results in decreased seizure activity (Tamargo et al., 2002). Similar results have also been achieved by intraventricular infusion. However, these techniques are not only invasive but can lead to several disadvantages such as susceptibility to infection, catheter obstruction, inadequate drug distribution (Scheld 1989), CNS toxicity associated with increased brain drug concentrations (Tamargo et al., 2002; Barcia et al., 2009; Pathan et al., 2009), changes in BBB physiology and transporter expression.

2.1.2 Biological tissue delivery

To obtain high concentration of drug into the brain is possible to use biological tissues. This technique requires an implant into the brain of a tissue that naturally secretes a therapeutic agent; and has been most extensively applied for the treatment of Parkinson's disease (Sladek and Gash 1988). However, the main problem connected to this strategy is that transplanted tissue cannot survive due to a lack of neovascular innervation. To enhance the survival and development of transplanted tissue, it is possible to use co-grafted cells engineered to release neurotropic factors with cells engineered to release therapeutic (Lu et al., 2014). Moreover, enhanced vascularization and microvascular permeability in cell-suspension embryonic neural grafts relative to solid grafts has been demonstrated (Leigh et al., 1994).

2.1.3 BBB disruption (BBBD) strategies

The invasive techniques include also BBB disruption strategies (BBBD). Several of these strategies have been investigated obtaining encouraging results. The following are reported the most important BBBD strategies.

Convection-enhanced delivery (CED)

Using the convection-enhanced delivery (CED) technique is possible the administration of therapeutic molecules directly into targeted brain parenchyma or tissue **(Figure 5).** One or more catheters are stereotactically placed into the brain through cranial burr holes and therapeutic agents are subsequently administered by microinfusion pump (DiMeco et al., 2002; Cunningham et al., 2008). CED shows unique characteristics for CNS delivery (Bidros and Vogelbaum 2009). This technique provides effective drug concentration, without molecular weight limitation, in the region of structure where the catheter is placed. Furthermore, CED does not produce measurable increases in intracranial pressure or cerebral edema (Chen et al., 1999).

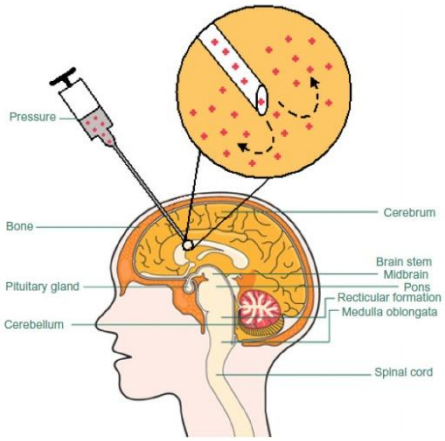

Figure 5: Convection-enhanced delivery. Compunds are infused through a cannula inserted into the target by a microinfusion pump. Continuous positive pressure provides to push the molecules further away from the cannula lip. Lu et al., 2014. *Int J Nanomedicine* 9: 2241–2257.

Osmotic BBBD strategy

Transient opening of the BBB can occur by intra-arterial infusion of hypertonic solutions such as those containing arabinose or mannitol (Neuwelt 2004; Kroll and Neuwelt 1998; Kroll et al., 1998). Hyperosmotic mannitol solution induces the shrinkage of cerebrovascular endothelial cells, resulting in a disruption of inter-endothelial TJs for several hours and increase of the BBB permeability (Rapoport SI and Robinson PJ 1986). Ca^{2+}-mediated contraction of the actin cytoskeleton may be facilitated these vascular events (Rapoport 2000). *In vivo* studies shown that administration of a Na^{+}/Ca^{2+} exchanger blocker can prolong osmotic opening of the BBB and increase sucrose permeability across the BBB (Bhattacharjee et al., 2001). Since its initial description, osmotic BBBD strategy has been used in preclinical and even clinical studies (Kraemer et al., 2001; McAllister et al., 2000) to improve delivery of CNS therapeutics. In particular, 25% mannitol solution improved brain penetration of chemotherapeutics used to treat malignant primary or metastatic brain tumors (Haluska and Anthony 2004). Besides mannitol, hyperosmolar solutions of urea, lactamide, arabinose, saline and radiographic contrast agents can be used to transiently open the BBB. Although effective at increasing permeability of therapeutics into the CNS, there are several drawbacks to this technique. However, studies have suggested that osmotic BBBD strategy has several disadvantages. Different variables, such choice of anesthetic, can dramatically affect the degree of BBB opening (Remsen et al., 1999). Moreover, the transient opening of the BBB results in an increase intracranial pressure and brain edema (Rapoport 2000; 2001). Finally, the BBB disruption leaves the CNS vulnerable to infection from circulating pathogens or allows entry of plasma proteins, thus triggering neuronal cell apoptosis (Pardridge 2012; Pathan et al., 2009).

Ultrasound (US)-mediated BBBD strategy

US-mediated BBBD is non-invasive disruptive technique used to transiently increase BBB permeability and enhance delivery of therapeutics into the brain. US consists of pressure waves having frequencies of 20 kHz or greater that pass through the skull and brain tissue and are concentrated onto small target area in the brain (Burgess and Hynynen 2013). Microbubbles, intravenous administrated and used as a contrast agent (Blomley et al., 2001), greatly reduce the acoustic energy required to open the BBB (Hynynen et al., 2001) **(Figure 6)**. Seems that the BBB opening is caused by expansion and contraction of these microbubbles during sonication, that stretches the blood vessel walls (Burgess and Hynynen 2013); this phenomena is called acoustic cavitation. The BBB opening is transient and reverses itself as early as 6 hours following US without tissue damage associated (Mesiwala et al., 2002). For several decades it was believed that to perform US treatments in the brain the skull bone had to be removed (Guthkelch et al., 1991). Theoretical and experimental studies have demonstrated that it is feasible to achieve focal, trans-skull focused US (FUS) exposure of brain tissue by using large surface area phased arrays (Pernot et al., 2003; Aubry et al., 2003). Recently, different studies using US-mediated BBDN strategy to deliver drugs to the targeted regions in the brain have shown encouraging results (Hynynen et al., 2004). In rats US mediated BBBD improved delivery of natural killer (NK) cells to cerebral tumor sites (Alkins et al., 2013). In a breast cancer metastasis model, US effectively enhance permeability of BBB and of blood tumor barrier, thus improving CNS delivery of the anti-cancer drug trastuzumab. Improved drug delivery allowed increasing survival time and decreasing brain tumor volume (Park et al., 2012). Moreover, electrical stimulation has also been shown to increase BBB permeability and improve drug delivery to the brain. Electrical stimulation of postganglionic parasympathetic fibers of the sphenopalatine ganglion (SPG) increases BBB permeability to FITC-dextran, as consequent of an increase in BBB permeability (Yarnitsky et al., 2004). Delivery of chemotherapeutic agents (etoposide and HER2 monoclonal antibody), was also improved by the use of electrical stimulation of postganglionic parasympathetic fibers of the SPG (Yarnitsky et al., 2004). Stimulation of the SPG has also been found to induce reperfusion and BBB protection in a rodent stroke model (Levi et al., 2012).

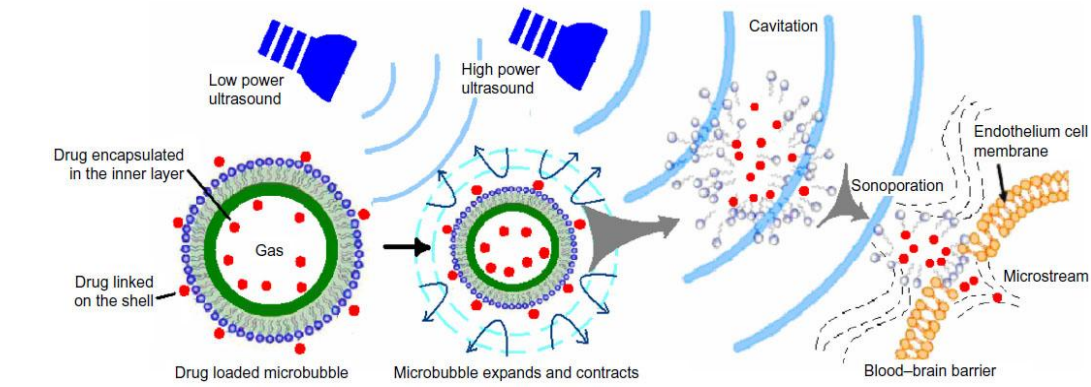

Figure 6: Ultrasonic microbubbles for drug targeted delivery. Lu et al., 2014. *Int J Nanomedicine 9: 2241–2257.*

Biochemical BBBD strategy

Several vasoactive molecules, including bradykinin, leukotrienes, and histamine, can selectively increase the permeability in abnormal brain capillaries (Cloughesy and Black 1995) Normal brain capillaries inactive these vasoactive compounds by an "enzymatic barrier". In contrast, this enzymatic barrier is lost in some disease conditions, and the vasoactive compounds can selectively increase permeability of abnormal capillaries. Intracarotid infusion of vasoactive agents can be used to improve drug delivery to diseased tissue. Biochemical BBBD is a less invasive than osmotic BBBD strategies and more reliable because it mainly affects the diseased vasculature (Lu et al., 2014).

2.2 Noninvasive techniques

Noninvasive techniques to improve the delivery into the brain include chemical and biological approaches and colloidal drug carriers.

2.2.1 Chemical approaches

Chemical approaches consist in transformation of chemical structure of drugs to improve their physicochemical properties and thus change their functionalities. Since lipid solubility is an important parameter in passive diffusion across the BBB, lipid groups can be added to the polar ends of drug molecules to assemble into lipophilic analogs, resulting in improved cerebrovascular permeability (Lu et al, 2014). However, this approach has different limitations. In particular, lipophilic analogs have low selectivity, poor tissue distribution and can affect the

rate of oxidative metabolism by cytochrome P-450 enzymes and other enzymes. Consequently the lipophilic analogs might cause the change of drug pharmacokinetic parameters (Bodor and Buchwald 1999).

An alternative to lipophilic analogs are prodrugs, pharmacologically inactive compounds that result from transient chemical modifications of biologically active compounds (Namba et al., 1998). With chemical modification the drug becomes more lipophilic. Morphine, a prodrug, cannot enter the CNS by itself but only via acetylation of both hydroxyl groups (Pardridge 1988). Moreover, prodrug approaches were explored for a variety of acid-containing drugs like GABA, levodopa, niflumic acid, valproate or vigabatrin (Savolainen et al., 2002). Flurbiprofen, neuroprotective agent in Alzheimer's disease, conjugated with lipoamino acids (LAA) to obtain amphiphilic derivatives with a membrane-like character shown good interaction and penetration through BBB (Pignatello et al., 2007). However, it should be considered that prodrug method may alter the original tissue distribution, the efficacy and toxicity of the parent drug (Lu et al., 2014).

The chemical drug delivery system (CDS) is another chemical approach explored to improve the drug delivery across the BBB (Bodor and Buchwald 1999). Different from the prodrug, a CDS typically requires only a single activation step. There are three types of CDS mostly investigated: site-specific enzyme-activated CDS; enzymatic physicochemical CDS; and receptor-based CDS. Among CDS, 1,4-dihydro-N-methylnicotinic acid (dihydrotrigonelline) increases the brain distribution of several drugs (Bodor and Buchwald 1999; Bewster et al., 1997; 1994; Wu et al., 1990; Sikic et al., 1997; Bodor and Buchwald 2002).

Alternately to these techniques, is possible to use a "molecular packaging" strategy. In this approach the drug is modified following three goals: increase its lipophilicity to promote passive transport, improve its enzymatic stability to prevent premature degradation, and exploit of the lock-in mechanism to provide targeting. In this way the peptide unit is a part of a bulky molecule, dominated by the groups that prevent recognition by peptidases and direct BBB penetration (Dwibhashyam and Nagappa 2008). DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) and thyrotropin-releasing hormone are two of peptide drugs modified with molecular packaging strategy for brain delivery (Petri et al., 2007; Bodor et al., 1992).

Finally, in order to increase the drug penetration of into the CNS, efflux transporters inhibitors have been developed. The first generation of P-gp inhibitors required extremely high doses to inhibit P-gp transport activity resulting in toxicity and unwanted pharmacokinetic interactions (Thomas and Coley 2003). Recently it was discovered that basal activity levels of P-gp are regulated via signaling through the sphingosine-1-phosphate receptor (S1PR). Animals treated with S1PR antagonists exhibit increased brain uptake of radiolabeled paclitaxel, verapamil, and loperamide, demonstrating reduced P-gp activity *in vivo* (Cannon et al., 2012). Moreover, polaxmers has also shown to enhance the permeability of several drugs on *in vitro* model of BBB by inhibiting drug efflux transporters (Kabanov et al., 2003; Litman et al, 2003; Sauna and

Ambudkar 2001). Newer-generation specific P-gp inhibitors such as, tariquidar 13 zosuquidar 12 and elacridar 11 have been shown an improved side effects profile (Breedveld et al., 2006). However, the drawback of this strategy is that with inhibition of ABC transporters also toxic substances can move freely into the brain (Wang et al., 2010).

2.2.2 Biological approaches

Several biological approaches to improve drug delivery across the BBB are investigated. They are based on the knowledge of physiological and anatomical mechanisms of BBB transport.

Cell-penetrating peptide (CPP)-mediated drug delivery

Cell-penetrating peptides are peptides containing a sequence of highly basic amino acids and thus positively charged. CPPs can transport tagged molecules across the cell membrane, into the cytoplasm and to the nucleus (Huwyler et al., 1996; Temsamani and Vidal 2004) via a receptor independent mechanism. Among the CPPs, most known are HIV-1 (human immunodeficiency virus [HIV] type 1) trans-activating transcriptional activator (TAT) peptide, antennapedia, herpes simplex virus (HSV) type-1 transcription factor (HSV VP-22) peptide, and penetratin (Huwyler et al., 1996). TAT peptide is a relatively small polypeptide of 86 amino acids with a cysteine rich region. It is able to migrate from the quiescently infected cells, where it is produced, to the uninfected cells and initiate viral replication. TAT protein contains the sequence known as protein transduction domain that is responsible for this translocation (Zheng et al., 2005) The basic region of TAT peptide consisting of two lysine and six arginine residues is essential for efficient cellular uptake (Vives et al., 1997). TAT peptide, interacting with the negatively charged phospholipids of plasma membrane, can permeabilize the cell by forming an inverted micelle by destabilization of phospholipid bilayer (Derossi et al., 1996). Different studies have proposed that cellular uptake of TAT peptide occurs by endocytosis and macropinocytosis. Along with efficient delivery of molecules, the main advantage of TAT coupling is preservation of biological activity of the coupled molecules. Moreover, TAT can facility the delivery of biomacromolecules through the BBB (Schwarze et al., 1999). Therefore, generally, the size of the molecule transported is not a rate-limiting factor.

Viral vectors

Viral vectors have been proposed for gene delivery at specific sites in the brain. They can be injected directly into the cerebral lateral ventricles and are delivered throughout the CNS (Kravcik et al., 1999) or injected at multiple sites to cover a large volume. Several virus classes have been studied as vectors for gene CNS delivery (Kumar et al., 2009) such as HSV (Wolfe D

et al., 1999), retrovirus (Cepko et al., 1998), lentivirus (Geraertes et al., 2006), simian virus (Louboutin et al., 2007) recombinant AAV (adeno-associated virus) (Mori et al., 2004), and helper-dependent adenovirus (Cregan et al., 2000). Moreover, agents such as mannitol (Lindgren et al., 2000) and heparin (Misra et al., 2003) have also been tested to improve the distribution of vectors.

Recently, various types of vectors have been constructed by using the combination of two or more viral elements or gene sources. Among these types of vectors there are: HSV/AAV hybrid amplicon vectors (Costantini et al., 1999), adenovirus/AAV hybrid vectors (Recchia et al., 1999), HSV/Epstein-Barr virus (EBV) and HSV/EBV/retrovirus hybrid amplicon vectors (Oehmig et al., 2004) and adenovirus/retrovirus hybrid vectors (Feng et al., 1997).

Although this strategy has generally demonstrated satisfactory results for CNS delivery, it is not without disadvantages. The principal limitations to use viral vector are the unwanted deleterious immune response, the changes in the properties of delivered virus due to endogenous recombination, and the mutagenic behavior leading to oncogenesis (Kumar et al., 2009).

Receptor/vector-mediated delivery of chimeric peptides

In receptor/vector-mediated delivery strategy non transportable peptide pharmaceutical can be coupled to a transportable peptide or protein and pass through the BBB via receptor-mediated or absorptive-mediated transcytosis (Kang et al., 1994). After binding the receptor and bound drug are internalized, and then separated. The receptor travels back to the membrane while the drug diffuses freely through the endothelial cell or crosses the abluminal membrane and diffuses into the brain parenchyma. The monoclonal antibody OX26, which recognizes the transferrin receptor, has been used to improve CNS delivery of therapeutic peptides by receptor-mediated transcytosis (Scherrmann 2002; Pardridge et al., 2012) and to bypass efflux transporter such as P-gp. Digoxin, a P-gp substrate, when incorporated into OX26-immunoliposomes showed a significantly increase of uptake by RBE4 rat brain capillary endothelial cells (Huwyler et al., 2002). Moreover, in hCMEC/D3 cells uptake and transcytosis of immunoliposome-associated dyes are higher as compared to control liposomes (Markoutsa et al., 2011). Some studies have shown that conjugation of ANG1005, a novel paclitaxel derivate, to angiopep-2 which enhances transcytosis *in vitro* as well as *in vivo* (Demeule et al., 2008) resulted in increased *in vivo* uptake of radiolabelled ANG1005 in mice with brain metastases of breast cancer (Thomas et al., 2009).

In addition, also adsorptive-mediated transcytosis can be used to deliver drugs into CNS. Cationic proteins bind to the luminal membrane of capillary endothelial cells via electrostatic interactions with anionic sites on the membrane (Scherrmann 2002; Tamai and Tsuji 2000) and trigger endocytosis. In the endothelial cells the drug can act on its intracellular target or freely diffuse into brain parenchyma to carry out its pharmacological effect (Pardridge et al., 2012).

2.2.3 Nanosystem based delivery

With the advent of nanomedicine, in the recent years, nanosystems have been proposed as noninvasive methods to cross the BBB and deliver drug to CNS (Holmes 2013; Re et al., 2012). Nanosystems are colloidal carriers with a diameter of between 1 and 1,000 nm (Kreuter 2001) **(Figure 7)**. Many of these carriers can be transported across *in vitro* and *in vivo* BBB models by endocytosis and/or transcytosis (Provenzale et al., 2005; Kreuter 2004; Jallouli et al., 2007) and have demonstrated preclinical success for treatment of CNS disorders (Wong et al., 2012). Particle size, stability in circulation and surface affinity are important factors for brain distribution of nanosystems (Bhaskar et al., 2010). Moreover, brain delivery of nanosystems can be further improved by covalent conjugation to ligands such as antibodies, proteins, or aptamers to target specific tissues. The large surface-area-to-volume ratio of nanocarriers permits to attach multiple copies of a ligand and to considerably increase their binding affinity via the multivalent functionalization (Montet et al., 2006). The extensively studied colloidal drug carriers are polymeric micelles, liposomes, nanoparticles, and dendrimers.

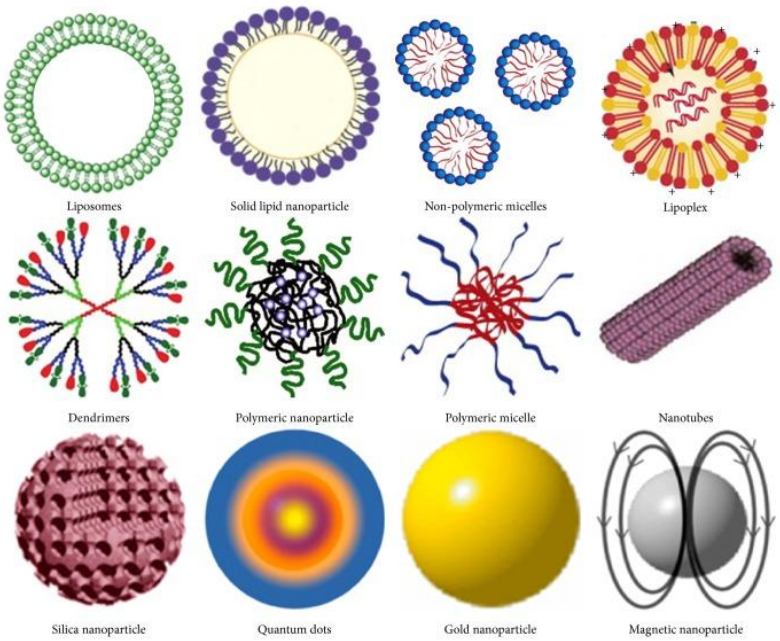

Figure 7: Different types of colloidal drug carriers. Graphical representation of the most commonly used nanocarriers for biomedical applications. Schemes are not in scale. Masserini 2013; *ISRN Biochem.* 2013: 238428.

Polymeric micelles

Polymeric micelles are made of amphiphilic polymers that aggregate in aqueous media to form spheroidal structures with hydrophilic shell and hydrophobic core (Mishra et al., 2010). Their stability can be increased by crosslinking between the shell or the core chains. Polymeric micelles can be designed to respond to external stimuli such as pH, light, temperature, ultrasound (Kabanov et al., 1992), thus triggering a controlled release of entrapped drugs. The main utilized polymer is pluronic type, block copolymer based on ethylene oxide and propylene oxide. Various studies have demonstrated that pluronic block copolymer micelles can inhibit drug efflux transporters such as P-gp and improve drug delivery to the CNS (Kabanov et al., 2002; Sabatier et al., 1991) without show toxicity to the BBB. Newer micelle systems are based on direct conjugation of drug molecule with targeting moiety to the amphiphilic portion (Kanwar et al., 2012; Pardridge et al., 1995). For example, transferrin-modified cyclo-(Arg-Gly-Asp-dPhe-Lys)-paclitaxel conjugate-loaded micelle enhances uptake by the brain microvascular endothelial cells *in vitro* and prolonges drug retention in glioma tumor in vivo without toxicity ohenomena. The small size and versatility of micelles make this delivery systems a promising tool for drug delivery into the brain (Masserini 2013).

Liposomes

Liposomes are lipid vesicles formed by one or more phospholipid bilayers and constituted of an aqueous core. They can transport hydrophilic drugs by encapsulating them in the aqueous core and amphiphilic and lipophilic drugs by solubilizing them in the phospholipid bilayer. Liposomes have as advantage to be easy to prepare, biocompatible, less toxic, and commercially available (Bhowmik et al., 2015). Conventional liposomes have low plasma circulation times due to elimination by the reticuloendothelial system. The circulation time of liposomes can be increased by decreasing the particle size (<100 nm) or by modification of the liposome surface with PEG (i.e., stealth liposomes). The liposomes have been already utilized to deliver drugs into the brain (Lai F et al., 2013), for the treatment of cerebral ischemia (Ishii et al., 2013) and brain tumours (Orthmann et al., 2012). Effective delivery of drugs like 5-fluorouracil (5-FU) and sodium borocaptate (Na210B12H11SH, BSH) to brain tumors has been achieved by liposome mediated delivery (Soni et al., 2008; Doi et al., 2008). Moreover, surface-modification methods have also been evaluated to improve the brain distribution for loaded liposomes (Pardridge 1999; Soni et al., 2008; Blasi et al., 2007). Modified liposomes such as p-aminophenyl-α-D-mannopyranoside (MAN), transferrin conjugated daunorubicin liposomes and trans-activating transcriptional peptide(TATp) modified liposomes have also been studied on *in vitro* and *in vivo* for targeting brain tumors (Ying et al., 2010; Gupta et al., 2007). Most recently are developed dual-targeting liposomes, encapsulating doxorubicin and conjugating

with both transferrin and folate. Transferrin to improve BBB transcytosis and folate to enhance the specificity for cancer cells in the brain. These kinds of liposomes were able to improve the therapeutic efficacy of brain glioma and were less toxic than the free-drug solution (Shilo et al., 2014; Gao et al., 2013).

Nanoparticles (NPs)

Polymeric nanoparticles (NPs) are colloidal particles formed of non-soluble polymers that are often biodegradable. NPs are divided into two classes: nanocapsules (core-shell structure) and nanospheres (matrix structure) and they are promising carriers for the CNS delivery of various drugs (Dechy-Cabaret et al., 2004). The drugs are encapsulated, entrapped, adsorbed, dissolved, or chemically linked to the surface of the NPs. The drug trapping method and the polymer structure decide drug characteristics and its release kinetics from the NPs (Juillerat-Jeanneret 2007). NPs can release the entrapped drugs by polymer degradation or by passive diffusion of the drug from the polymeric core. Polylactide homopolymers (polylactic acid) and poly(lactide-co-glycolide) (PLGA) are two classes of polymers approved by the Food and Drug Administration (FDA) for human use. These polymers offer several advantages for drugs delivery to CNS because they are biodegradable, biocompatible, and their NPs do not induce inflammatory response after injection (Dechy-Cabaret et al., 2004). Loperamide, tubocurarine, and doxorubicin are been transported in brain using NPs (Tosi et al., 2007). The encapsulation of drugs within NPs has minimized the efflux of the drug and improved its entry into the CNS. Moreover, hydrophilic and hydrophobic drugs can be entrapped in the NPs matrix and released in a sustained manner for several weeks. Functionalization of NPs by surface modification and conjugation with transporters of the BBB has been proposed to improve their transport efficiency into the brain (Bhaskar et al., 2010; Kanwar et al., 2012; Kulkarni and Feng 2011). Surface modification of NPs includes surfactant modification and antibody modification (Kanwar et al., 2012). For example, antibodies targeted to transferrin and insulin receptors can act as transporters across the BBB and deposit the drug molecules via receptor-mediated endocytosis. Conjugation with the transferrin receptor antibody can successfully deliver to the brain chemotherapeutic drugs like methotrexate (MTX) and proteins like basic fibroblast growth factor, nerve growth factor, and brain-derived neurotro phic factor (Pardridge et al., 1995; 1998). Moreover, several studies have been demonstrated that surface modification of NPs with polysorbate 80, a bio-adhesive surfactants, show enhanced brain specificity and efficacy compared with unmodified NPs (Kreuter et al., 1995; Steiniger et al., 2004; Wang et al., 2009). Another surfactant, poloxamer 188 (F68) has been found to increase the delivery of drug-loaded polybutylcyanoacrylate and PLGA NPs to the brain in rats (Petri et al., 2007; Gelperina et al., 2010). Probably these surfactants can enhance brain permeation by inhibition of the efflux action of P-glycoprotein and solubilization of cell membrane lipids of brain endothelial cells for

entry (Kulkarni and Feng 2011). NPs, surface-modified with polysorbate 80 and F68, adsorb apolipoprotein E, apolipoprotein B, or both, mimic low-density lipoproteins, and enter the brain by receptor-mediated endocytosis (Pandey et al., 2003). Many other molecules were conjugated to the NPs to enhance the passage through the BBB among them lactoferrin (Hu et al., 2009) and insulin (Shilo et al., 2014). However the NPs transport across the BBB may be decrease for intracellular accumulation at the endothelial cells as large aggregates. This issue can be addressed using the "shuttle mediated" transport strategy (Lu et al., 2014). Recent studies have demonstrated that conjugation of NPs with a viral fusion peptide (gH625) derived from the glycoprotein gH of HSV, the formation of NP aggregates within the cells is decreased, and improved BBB transport (Guarnieri et al., 2013).

Solid lipid NPs (SLNs)

Solid lipid nanoparticles (SLN) are lipid-based nanocarriers with solid hydrophobic lipid core, that is stabilized by surfactants and the drugs are dissolved or dispersed in this core (Kaur et al., 2008). They are made with biocompatible lipids and are generally of small size (40-200nm) to escape from the reticuloendothelial system (RES) (Pardeshi et al., 2012). The advantages of SLNs are their biocompatibility, drug entrapment efficiency, and the ability to provide a continuous release of the drug for several weeks (Mishra et al., 2010). Moreover, they can be modified on their surface to target molecules to the brain and limit RES uptake (Blasi et al., 2007). SLNs have been used to deliver into the brain many therapeutic agents, such as doxorubicin (Agarwal et al., 2011); docetaxel (Venishetty et al, 2013), quercetin (Dhawan et al., 2011); atazanavir (Chattopadhyay et al., 2008), and quinine (Gupta et al., 2007). Moreover, PEGylated SLNs have been widely investigated for brain drug delivery (Wong et al., 2007; Brioschi et al., 2007). Due to their lipidic nature SLNs and PEGylated SLNs are readily taken up by the brain tissues. They are less toxic than polymeric NPs for their biocompatible and biodegradable nature. In addition, their small size, circulation time prolonged, feasibility for large scale production and absence of burst effect makes them promising tools for CNS drug delivery (Kaur et al., 2008).

Dendrimers

Dendrimers are branched polymers that remind the structure of a tree. A dendrimer is typically symmetric around the core, with a structure tightly packed in the periphery and loosely packed in the core, leaving spaces for drug entrapping ability (Dhanikula et al., 2009). The most known molecule for synthesis of dendrimers is poly(amidoamine), or PAMAM. Dendrimers are highly versatile nanocarriers with a good potential in brain delivery (Sarin et al., 2008) and their

surfaces can be easily functionalized due to the availability of multiple reactive functional groups. Dendrimers complexed with transferrin-conjugated polyamidoamine have shown a significant increase in the brain uptake of DNA (deoxyribonucleic acid) (Huang et al., 2007). In another study, mannosylated polyamidoamine dendrimers loading lamivudine were evaluated to enhance the antiretroviral activity in HIV-infected MT2 cells. However, the main limitation of dendrimers-based drug delivery is their tendency to release the drug quickly, before the reach the targets (Wolinsky et al., 2008).

2.3 Alternative routes for CNS drug delivery

2.3.1 Intranasal delivery

Intranasal administration has emerged as an alternative route for noninvasive drug delivery to the CNS bypassing the cardiovascular system and BBB (Thorne and Frey 2001; Illum 2000; Mathison et al., 1998). The principal route for intranasal delivery is the olfactory nerve pathway **(Figure 8)**. Drugs via the olfactory nerve axons, can accumulate in olfactory bulbs (OB), and diffuse into the brain following adsorption from the nasal mucosa (Dhuria et al., 2010). Size and lipophilicity of drugs can act on the efficiency of CNS delivery following intranasal administration (Sakane et al., 1995). Several studies suggest that intranasal delivery is a promising approach to deliver nanostructured therapeutics to the brain. Intranasal administration of rivastigmine encapsulated in liposomes was evaluated for treatment of Alzheimer's disease, demonstrating a higher drug concentration and a longer half-life of drug in the brain compared to intranasal free drug or the oral route (Arumugam et al., 2008). In addition, intranasal delivery was explored as an alternative to administrate anticancer drugs for treatment of brain tumors. Intranasal administration of methotrexate results in higher drug brain concentrations with reduction of tumor weight by 80% compared to control (Shingaki et al., 2010). Nanostructured lipid carriers encapsulating valproic acid are tested in a rat electroshock model, administered via different routes (intranasal versus intraperitoneal). Nanoformulation provides significant protective effects against the electroshocks in both routes of administration but with a much higher brain to plasma drug concentration ratio in the intranasal administration (Eskandari et al., 2011). Different studies show the potential advantage of intranasal delivery over other systemic routes of administration. The advantages of intranasal delivery include systemic side effects greatly reduced, absence of degradation in blood (Banks 2004), drugs targeted to specific regions of brain (Falcone et al., 2014; Nonaka et al., 2012; 2008). However, there are also disadvantages for this delivery strategy such as reaction in the nasal cavity, leading to irritation of the cells and nerves present within the cavity (Gizurarson 2012), limited absorption across the nasal epithelium (Lochhead et al., 2012), due to degradation by the nasal mucosa (Illum L 2003), the small surface area for absorption (Gizurarson 2012), and distance of transport or

diffusion required to reach the target brain region (Meredith et al., 2015). Therefore, it is request an increased research activity in applying intranasal delivery to improve the knowledge on this alternative route for drug delivery to brain.

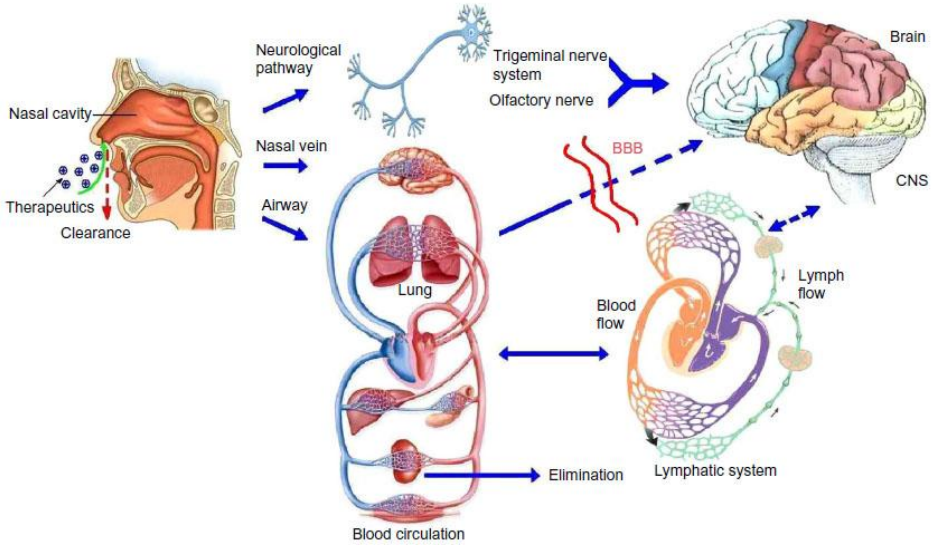

Figure 8: Olfactory and trigeminal pathways to the CNS. Lu et al., 2014. *Int J Nanomedicine 9: 2241–2257.*

2.3.2 Iontophoretic delivery

Recently, the iontophoretic technique to deliver drugs into the brain has been explored. This technique permits to deliver ionized molecules through the BBB by using an externally applied electric current (Jogani et al., 2008). Noninvasive and invasive iontophoretic methods have also been presented to improve the delivery of macromolecule agents to the brain under controlled manipulation (Lu et al., 2014).

3. LIPOSOMES FOR BRAIN DRUG DELIVERY

Since their discovery in the 1960s, the liposomes were considered promising nanocarriers for drug delivery. The most advantageous features of liposomes are their ability to incorporate and deliver large amounts of drugs and the possibility to decorate their surface with different ligands. Moreover, they are able to stabilize therapeutic molecules, overcoming obstacles to cellular and tissue uptake, and improve their biodistribution to target sites *in vivo* (Koning and Storm 2003; Metselaar and Storm 2005; Ding et al., 2006; Hua and Wu 2013). In this way they can enable effective delivery of encapsulated drugs to target sites while minimizing systemic toxicity associated. The application of liposomes as drug delivery systems has already had a major impact on many biomedical areas. Several liposomes are been approved for treatment of different pathologies while others are in clinical trials or are about to be put on the market. Due to their flexible physicochemical and biophysical properties, liposomes represent an attractive system to deliver drugs into the brain.

3.1 Physico-chemical characteristics of liposomes

Liposomes are vesicles composed of phospholipids consisting of one or more concentric lipid bilayers enclosing discrete aqueous core. Phospholipids are amphiphilic molecules with a hydrophilic head and two apolar hydrophobic chains that have a strong tendency to form membranes in aqueous solutions (Papahadjopoulos and Kimelberg 1973). The formation of these lipid bilayers is based on hydrophobic interactions, while van der Waals forces and hydrogen stabilize this structure. The final organization of lipids depends on their nature, concentration, temperature, and geometric form (Frolov et al., 2011).

Liposomes can deliver a variety of compounds, due to ability to entrap hydrophobic molecules into the bilayer membrane and hydrophilic molecules in the aqueous core (Koning and Storm G, 2003; Metselaar and Storm, 2005; Ding et al., 2006; Hua and Wu, 2013). Moreover, the large aqueous compartment and biocompatible lipid exterior allows the delivery several macromolecules, such as DNA, proteins and imaging agents (Ulrich 2002; Monteiro et al., 2014).

On the basis of number of lamellae, the liposomes are classified in unilamellar vesicles (ULVs) or multilamellar vesicles (MLVs). ULVs are characterized of one lipid bilayer with a large aqueous core and they are ideal to encapsulate hydrophilic drugs. MLVs are formed by two or more concentric lipid bilayers organized like an onion-skin, and they can entrap lipid-soluble drugs (Immordino et al., 2006). It has been hypothesized that ULVs and MLVs have different release kinetics. ULVs with a hydrodynamic diameter of 130 nm show a much faster release rate than MLVs with two to three lamellar bilayers and a hydrodynamic diameter of 250 nm (Betageri and Parsons 1992; Niven et al., 1991). The different release rate is due to the number

of phospholipid bilayer that the drug crosses before being released (Bozzuto and Molinari 2015).

Liposomes are usually within the size range of 10 nm to 1 μm or greater. On the basis of size, liposomes are classified into small unilamellar vesicles (SUVs), giant unilamellar vesicles (GUVs), and large unilamellar vesicles (LUVs) (Kaur et al., 2004; Mainardes et al., 2005; Elbayoumi and Torchilin 2010) **(Figure 9)**.

The modulation of lipid composition, size and charge can change the stability, pharmacokinetic proprieties and therapeutic efficacy of liposomes.

In particular, the addition of cholesterol to lipid bilayer can reduce the permeability and increase the stability of liposomes. The cholesterol, a hydrophobic molecule, interacting with the core of the membrane induces a dense packing of phospholipids and inhibits their transfer to high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Cholesterol can be also used to anchor other molecules to liposomes such as polyethylene glycol (PEG) that proved to extend the blood-circulation time while diminishing the uptake by the reticuloendothelial system (RES) (Allen 1995; Schechter 2002; Hosta-Rigau et al., 2013).

Moreover, to prolong the *in vivo* liposome circulation time, hydrophilic carbohydrates or polymers, such as monosialoganglioside (GM1) are included in liposome composition. GM1 decreases the blood proteins absorbed on the liposomal surface and increases the half-life of liposomes in the blood (Milla et al., 2012, Gabizon and Papahadjopoulos 1988). Also phosphatidylcholine with saturated fatty acyl chains and materials that stretch the transition temperature beyond 37°C permit to increase the liposomes stability (Bitounis et al., 2012).

The interaction with the cell membrane and the pathway of internalization of liposomes can occur in several ways and are strictly dependent on the physicochemistry of liposomes (Bozzuto and Molinari 2015). The liposome can be adsorbed into the cells membrane, its lipid bilayer is degraded by enzymes or by mechanical strain and the drug is released into the extracellular fluid and diffused through the cell membrane and cytoplasm. Otherwise, the liposome membrane can fuse with the plasma membrane of the target cell, and liposomal content is released into the cytoplasm. Another way of drug internalization is receptor-mediated endocytosis. In this process liposomes, with maximum diameter of 150 nm, are internalized and enzymatically processed in lysosomes. Finally, phagocytosis can also occur but involves liposomes of a diameter larger than 150 nm and specialized cells of the immune system, such as macrophages, monocytes, and Kupffer cells (Fanciullino and Ciccolini 2009; Torchilin 2011; Fanciullino et al., 2005). The mechanism and extent of liposome-cell interaction depend by the nature and density of the charge on the liposomes surface (neutral, positive, or negative). The neutral liposomes (without charge on the surface) are unstable, aggregate easily, do not interact significantly with cells and the drug is released in the extracellular space (Senior et al., 1991; Zhao et al., 2011).Whereas, the presence of charge induces electrostatic repulsion among liposomes, preventing their aggregation and flocculation. Moreover, charged liposomes can

interact much more with cells (Buzzuto and Molinari 2015). Negatively charged liposomes, generally constituted by anionic lipids, are less stable than neutral and positive liposomes. When injected in the blood circulation, the negative liposomes rapidly interact with the biological system subsequently to their opsonization with complement and other circulating proteins (Harashima et al., 1998; Miller et al., 1998; Cullis et al., 1998) producing toxic effects, such as pseudoallergy (Campbell 1983). However, in recent years, several studies have demonstrated that the negatively charged liposomes diffuse to the dermis and the lower portion of hair follicles through the stratum corneum and the follicles much more quickly than the positive liposomes. Therefore, negative liposomes would contribute to increased permeation of drugs through the skin (González-Rodríguez et al., 2011; Ogiso et al., 2001).

Finally, cationic liposomes (CLPs) consist of natural neutral phospholipids and positively charged lipids. They are preferentially internalized via endocytic pathway and are typically used for gene delivery, based on the electrostatics between positively charged lipids and negatively charged nucleic acids (Felgner et al., 1987). The presence of a positive charge on liposomes improves their interactions with the glycoproteins of the endothelial cell membranes (Campbell et al., 2002). It has been demonstrated that drugs such as paclitaxel, Dox, and oxaliplatin entrapped in the CLPs improve their antitumor efficacy by reducing functionality of the tumor microvasculature (Strieth et al., 2004; Wu et al., 2007).

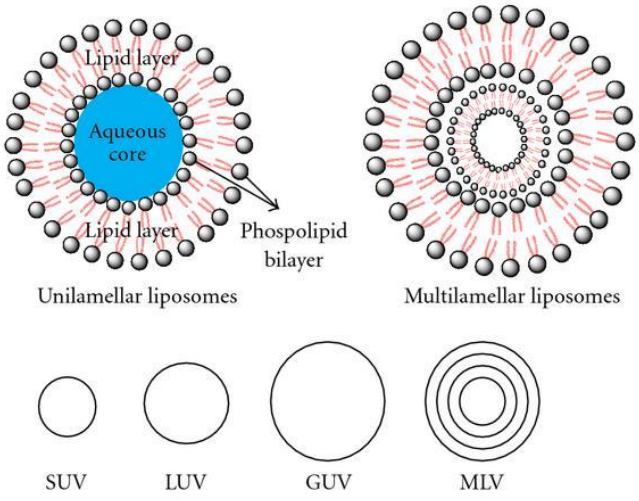

Figure 9: Schematic representation of different liposomal structures. SUV: ~ 20 to 100 nm; LUV: ~ 100 nm to 400 nm; MLV: ~ 200 to 3 µm, GUV: > 1 µm. Gyan et al., 2011. *J Drug Deliv*. 863734.

3.2 Methods for liposomes preparation

The methods to prepare liposomes are many different. Among these the most used method is the thin-film hydration or Bangham method (Bangham et al., 1965; New 1990). This technique consists in the dissolution of the lipid in an organic solvent, evaporation of solvent, and dispersion of the obtained lipid film in aqueous media. The drug can be entrapped in the aqueous media if it is hydrophilic or in the lipid film if it is lipophilic. The disadvantages of this method are the low encapsulation efficiency of water-soluble drugs (5%-15%) and production of large and nonhomogeneous MLVs that require sonication or extrusion to produce in homogeneous small ULVs.

Methods with higher encapsulation efficiency are the reverse-phase evaporation and solvent-injection that provide hydration of the lipids directly from an organic solvent, and achieve an aqueous suspension of MLVs and ULVs, respectively (Szoka and Papahadjopoulos 1978; Batzri and Korn 1973; Deamer and Bangham 1976). Moreover, the dehydration-rehydration technique provides high drug-encapsulation efficiency by inducing the fusion of preformed vesicles by means of dehydration and controlled rehydration (Kirby and Gregoriadis 1984).

Generally, liposomes made with the aforementioned methods require postformation processes to modulate their size, lamellarity, and homogeneity. Among these postformation processes are membrane-extrusion, sonication, and high-pressure homogenization. The membrane-extrusion technique decreases the size of the liposomes by passing them through a membrane filter with a defined pore size (Hope et al., 1985; Berger et al., 2001; MacDonald et al., 1991). Sonication reduces the size of the vesicles and gives energy to lipid suspension by an ultrasonic irradiation to the suspensions of MLVs (Woodbury et al., 2006). The high-pressure homogenization permits the subdivision of vesicles into smaller sizes using a special homogenizing valve (Bachmann et al., 1993).

The industrial-scale production of liposomes has led to extend the number of preparation methods. Among these, the microfluidic-based method permits a precise control of liposome size and size distribution (Yu et al., 2009) manipulating liquid flows in channels with dimensions of 10 to 100 of micrometers (Stone et al., 2004; Whitesides 2006). This method may be optimized to support real-time generation of purified liposomes with high concentrations of drugs and minimal reagent waste (Hood et al., 2014).

After their preparation, it is very fundamental to investigate the liposomal structures and their stability in different environments, and several techniques are available for these purposes. Organic electrochemical transistors (OECTs) are sensitive devices for detecting liposomes on a wide dynamic range down to 10-5 mg/mL, thus matching the needs of typical drug-loading/drug-delivery conditions. OECTs are able to sense and discriminate successive injections of different liposomes, and so could be good candidates in quality-control assays or in the pharmaceutical industry (Tarabella et al., 2013).

It is also extremely important to evaluate parameters such as shape, lamellarity, size, and surface features that strongly influence *in vitro* and *in vivo* performance of liposomes. Liposome shape can be evaluated using electron microscopy techniques; liposome lamellarity by using negative staining and/or freeze-fracturing for transmission electron microscopy (TEM) and P-31 nuclear magnetic resonance analysis (Lasic 1991). The size and size distribution of liposomes are characterized using several methods. Among these the most widely applied include dynamic light scattering (DLS), size-exclusion chromatography, field-flow fractionation (FFF) (Ostrovsky 1993; Palmer et al., 2003; Gabielle-Madelmont et al., 2003; Moon et al., 1993) and various microscopy techniques, such as cryo-TEM (Ruozi et al., 2011; Frederik and Hubert 2005).

DLS is a simple and rapid method to measure the time-dependent fluctuations in the intensity of scattered light. These fluctuations are due to collisions between solvent molecules and liposomes suspended that are undergo random Brownian motion. An analysis of the intensity fluctuations permit to know the distribution of the diffusion coefficients of the liposomes, which are converted into a size distribution using established theories (Bozzuto and Molinari 2015).

Electron microscopy techniques such as cryo-TEM and TEM using freeze-fracturing, provide exact information about the profile of the liposome population over the whole range of sizes (Ruozi et al., 2011). Atomic force microscopy provides information with high resolution on the three-dimensional profile of liposomes without removing them from their native environment (Jass et al., 1993; Ruozi et al., 2005).

Size-exclusion chromatography is used to separate and quantify liposome populations by exploiting the time-based resolution of hydrodynamic size. The process of separation is based on large-particle elution before that of smaller particles (Gabielle-Madelmont et al., 2003; Ruysschaert et al., 2005).

Field-flow fractionation (FFF) is a separation technique based on the laminar flow of particles in a solution, using semipermeable membrane. FFF separates liposomes based on size, and can separate materials over a wide colloidal size range while maintaining high resolution (Korgel et al., 1998).

Unfortunately, many of these techniques are very expensive and require specific equipment. The discovery of real-time, highly sensitive, and low-cost monitoring systems of several parameters of liposomes could be very useful both for pharmaceutical manufacturing and for quality-assurance assays applied to liposomal formulations.

3.3 Liposomes as nanomedical tools

Due their flexible physicochemical and biophysical properties, and their biocompatibility and biodegradability (Immordino et al., 2006), the application of liposomes in medicine offers promising prospects for novel and effective treatments in a wide range of diseases **(Figure 10)**.

Liposomes have been already utilized to deliver several therapeutic molecules and diagnostic agents (Hua and Wu 2013). Clinical studies have shown that encapsulation of drugs in liposomes leads to a change in toxicity profiles.

Conventional liposomes are the first generation of liposomes to be developed and they consist of a lipid bilayer composed of cationic, anionic, or neutral (phospho)lipids and cholesterol, which encloses an aqueous core. They have shown to improve the therapeutic index of encapsulated drugs, such as doxorubicin and amphotericin (Gabizon et al., 1982; Koning and Storm 2003; Metselaar and Storm 2005; Ding et al., 2006; Hua and Wu 2013). Moreover, these liposomes reduce the toxicity of compounds *in vivo*, by modifying pharmacokinetics and biodistribution to enhance drug delivery to diseased tissue in comparison to free drug. However, they were rapidly eliminated from the bloodstream due to opsonization of plasma components and uptake by fixed macrophages of the reticuloendothelial system (RES), mainly in the liver and spleen (Hua and Wu 2013), limiting their therapeutic efficacy (Gabizon et al., 1991, 1994). The opsonization of liposomes occurs by adsorption of plasma proteins such as immunoglobulin, lipoproteins, fibronectin, and/or complement proteins onto the phospholipid membrane (Ishida et al., 2001a; Chrai et al., 2002). The liposomes uptake by RES is due to fenestrations in their microvasculature (pore diameters from 100 to 800 nm) that permit the extravasation and subsequent removal of most drug-loaded liposomes (50-1000 nm in size) (Sapra and Allen 2003). Adding of polyethylene glycol (PEG) to the liposomes surface is the common strategy to increase their circulation times and prevent removal by the RES (Oku and Namba 1994; Ishida et al., 2001a). These modified liposomes are known as stealth liposomes. The presence on the surface of PEG, a hydrophilic polymer, creates a steric barrier that inhibits both electrostatic and hydrophobic reaction with plasma proteins and/or cells, thus reducing *in vivo* opsonization and uptake by the RES. This strategy not only prolongs blood circulation, but also decreases side effects (Torchilin et al., 1992; Northfelt et al., 1996; Ishida et al., 2001b). Steric stabilization strongly influences the pharmacokinetics of liposomes (Gabizon et al., 1993), with an increase of half-lives varying from 2 to 24 h in mice and rats, and as high as 45 h in human, depending on the particle size and the characteristics of the coating polymer (Allen 1994; Moghimi and Szebeni 2003). However, liposomes modified with PEG shown a reduction in the ability to interact with the intended targets (Willis and Forssen 1998; Ulrich 2002).

Modifications of liposomal formulations are constantly investigated to increase efficacy, reduce RES clearance and toxicity, this includes changes in lipid composition, charge, and the addition of surface coatings and ligands (Hua and Wu 2013; Monteiro et al., 2014). More recent strategies to improve the conventional or stealth liposomes involve active targeting, triggered release, charged lipids, and multi-functional formulations (Puri et al., 2009; Allen and Cullis 2013; Bozzuto and Molinari 2015; Sercombe et al., 2015).

The conjugation of targeting ligands to the surface of liposomes has been extensively investigated for several biomedical applications, and particularly by intravenous and

intraperitoneal injection (Torchilin 1994; Vingerhoeds et al., 1994; Willis and Forssen 1998; Noble et al., 2004; Deshpande et al., 2013; Hua and Cabot 2013; Rip et al., 2014; Hua et al., 2015). Targeting ligands are used to increase the specificity of delivery of entrapped drug to diseased tissues and cells, and reduce the uptake by non-target tissues. The therapeutic advantage of this strategy is still debated (Ferrari 2005; Puri et al., 2009; Riehemann et al., 2009). Several studies have demonstrated that ligand-targeted liposomes can enhance the uptake and efficacy in diseased tissue in comparison to non-targeted liposomes *in vivo* (Vingerhoeds et al., 1994; Puri et al., 2009; Allen and Cullis 2013; Kraft et al., 2014). For example, in a rodent model of musculoskeletal pain, the attachment of ICAM-1 monoclonal antibodies to the surface of loperamide-encapsulated liposomes increases their efficacy and localization to peripheral inflammatory tissue (Hua and Cabot 2013). Moreover, the coniugation of folate to liposomes enhance their biodistribution in folate-expressing tumors in a murine model (Gabizon et al., 2003). The doxorubicin-loaded anti-HER2 immunoliposomes have shown improved therapeutic results in comparison to all other control groups, including free doxorubicin, non-targeted liposomal doxorubicin and recombinant anti-HER2 Mab trastuzumab (Park et al., 2002). The enhanced anti-tumor efficacy was due to the marked difference in pharmacodynamics of the targeted liposomes *in vivo*, which are able to improve intracellular drug delivery to HER2-overexpressing cancer cells (Kirpotin et al., 2006). Therefore, the use of targeting ligands probably can enhance therapeutic efficacy by increasing receptor-mediated uptake of drug-encapsulated liposomes into target cells, subsequent to their accumulation in the diseased tissues (Kirpotin et al., 2006; Puri et al., 2009). However, the optimal targeting ligand density on the surface of liposome still is unknown, and likely depends from characteristics of the molecular target (Puri et al., 2009; Hua and Wu 2013; Kraft et al., 2014).

Modifications of charge of liposomes have also shown interesting results (Bozzuto and Molinari 2015). The surface charge of liposomal systems can influence their electrostatic interaction with components in the gastrointestinal (GI) tract after oral administration, and should confer selectivity to diseased tissue (Hua et al., 2015). In particular, cationic liposomes are not only able to transport large, charged structures (Felgner et al., 1987; Campbell et al., 2002, 2009; Kunstfeld et al., 2003; Wu et al., 2007) but they also interact with tumor vessels that overexpress negatively charged functional groups on the angiogenic endothelial cell membrane (Ran et al., 2002). However, these types of liposomes may be subject to electrostatic interactions and subsequent binding with other charge-modifying substances in the circulation or during GI transit (Hua et al., 2015).

Moreover, to improve the therapeutic efficacy of liposomes is possible to use triggering modalities for site-specific release of drugs (Bibi et al., 2012). Through the use of specific lipid compositions and coatings, several strategies of triggers were tested including remote triggers (e.g., ultrasound, temperature, magnetic, and light) and local triggers specific to the target site (e.g., pH and enzymes) (Guo and Szoka 2003; Andresen et al., 2004; Ponce et al., 2006; Bibi S

et al., 2012). In particular, external hyperthermic trigger to release therapeutic molecules from liposomes, such as ThermoDox®, appears to be the most promising strategy (Needham et al., 2000). Thermosensitive liposomes, modified with temperature-sensitive lipids and/or polymers, are stable and retain their contents at physiologic temperatures, but upon heating they undergo a phase change and became more permeable, causing the release of their cargo (Kono 2001). Temperature-sensitive liposomes have been applied in preclinical and clinical studies in combination with heat-based thermal therapies, including ultrasound hyperthermia, radiofrequency ablation, and microwave hyperthermia (Gasselhuber et al., 2010; Poon and Borys 2009; Dromi et al., 2007; Hauck et al., 2006). Recently, doxorubicin-encapsulated in cationic thermosensitive liposomes (CTSL) for tumor targeting have shown three-fold higher accumulation at the target site compared to the thermosensitive liposomes (Dicheva et al., 2013, 2014). However, even if the translation of these drug delivery systems into the clinic has not yet been reached, due to issues on therapeutic efficacy and potential toxicity (Bibi et al., 2012; Allen and Cullis 2013), they represent a promising strategy.

Furthermore, several studies have investigated complex multi-functional liposomes to develop more efficient drug delivery systems. In particular, theranostic liposomes have generated much interest as it is both a therapeutic and diagnostic tool. An ideal theranostic delivery system includes the nanoparticle, targeting ligand, imaging component, and therapeutic agent. Due to their characteristics (size, hydrophobic and hydrophilic character, biocompatibility, biodegradability, low toxicity, and immunogenicity) the liposomes represent a valid platform for theranostic nanomedicine. Several studies have demonstrated effective diagnostic imaging and therapeutic delivery of encapsulated drugs *in vivo* with theranostic liposomes, especially in various cancers (Charron et al., 2015; Cole and Holland 2015). Liposome-nanoparticle hybrids, designed by embedding, encapsulation, or conjugation of nanoparticles onto various types of liposomes, have shown theranostic potential. Such as Doxloaded liposome with lipid bilayer-embedded quantum-dot vesicle hybrids capable of chemotherapy, for cytotoxic activity of Dox, and optical imaging for embedded quantum dots (Al-Jamal and Kostarelos 2011). Moreover, conjugated ECl-GLuc to nickel-chelating liposomes (ECl-GLuc-liposome) have shown significant targeted drug delivery and bioluminescence imaging in SKOv3 cells *in vitro* and in ErbB2-overexpressing metastatic ovarian tumors *in vivo* in a murine model (Han et al., 2014). ECl-GLuc is a recombinant protein obtained by fusing the ECl peptide, an artificial ligand of ErbB2, with Gaussia luciferase (GLuc) (Sercombe et al., 2015). Although efficient, the translation of these complex multi-functional liposomes to the clinic would need to show significant therapeutic advantage in comparison to other therapeutic strategies, due to the added costs and complexities required in the manufacturing process. Furthemore, these systems will face the potential mismatch between the doses required for the effective use of imaging and therapeutic components in the patients (Teli et al., 2010).

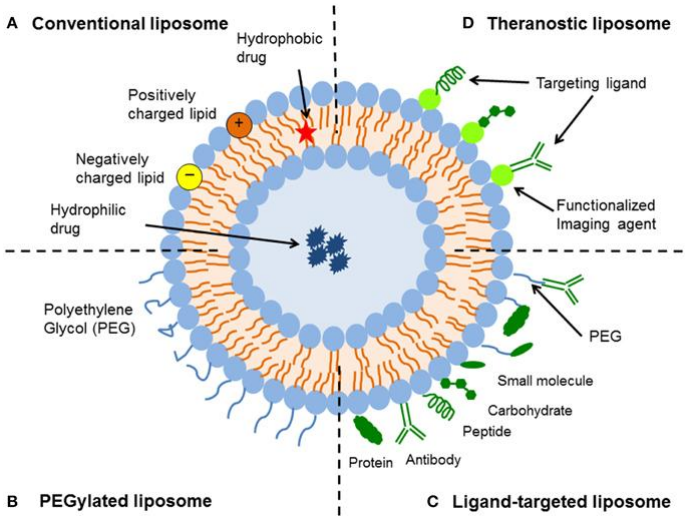

Figure 10: Different types of liposomes for drug delivery. (A) Conventional liposome; (B) PEGylated liposome; (C) Ligand-targeted liposome; (D) Theranostic liposome. Sercombe et al., 2015. *Front Pharmacol.* 6:286

3.4 Liposomes clinically approved or in clinical trials

Currently, many liposomal products are on the market and many others are still in the various stages of clinical trials (Chang and Yeh 2012) **(Table 1)**. Some of them are PEGylated liposomes just like the first pharmaceutical product in a liposomal formulation FDA approved, used to deliver doxorubicin (Ning et al., 2007). Often in combination with other drugs, PEGylated liposome Dox (PLD) is used for treatment various types of cancer including AIDS-related Kaposi's sarcoma, leukemia, and ovarian, lung, breast, bone, and brain cancers, and it represent also an effective alternative to conventional doxorubicin in patients with pre-existing cardiac dysfunction (Schmitt et al., 2012). PLD decreases the uptake and clearance by the RES, increases half-life in the serum and plasma thus resulting in an accumulation of drug in the tumor tissue, rather than in healthy tissues, and moreover reduces cardiac muscle cell toxicity (Rahman et al., 2007). Combination therapies of PLD and other drugs, such as bortezomib to treat relapsed or refractory multiple myeloma, have recently received FDA approval (Ning et al., 2007).

Moreover, conventional and cationic liposomal-based drugs have also been FDA approved and are currently on the market. Among them are liposomal amphotericin B for anti-fungal prophylaxis (Chandrasekar 2008), daunorubicin for the treatment of leukemia and solid tumors, verteporfin for treatment of macular degeneration, cytarabine or cytosine arabinoside for

neoplastic meningitis and lymphomatous meningitis, and morphine sulfate for pain management (Chang and Yeh 2012; Jahn et al., 2015). Advantages of these marketed drugs are ability to encapsulate compounds with different lipophilicities, by protecting them from biodegradation, and reduced toxicity by increased vasculature permeability/accumulation at the target tissue (Immordino et al., 2006; Chang and Yeh 2012; Allen and Cullis 2013). However, many of these drugs are still undergoing clinical trials to test their effects of dose escalation and therapeutic efficacy (Chang and Yeh 2012). For example, liposomal amphotericin B is in a prospective Phase II trial to investigate the safety and tolerability of high doses (Giannella et al., 2015).

In addition to these, many liposomal-based drugs are in various stages of clinical development. PEGylated liposome currently in Phase I trials is PEPO2, an irinotecan-encapsulated liposomes used for treatment of advanced refractory solid tumors (Chang et al., 2015). Camptothecin, formulated in PEGylated stealth liposomes, is also in Phase I trials to treat ovarian cancer (Zamboni et al., 2009). Moreover, irinotecan SN-38 is in Phase I/II to treat colorectal cancer (Zhang et al., 2004; Suenaga et al., 2015) and a liposomal-based all-trans-retinoic acid (ATRA) in Phase II for treatment of acute promyelocytic leukemia and hormone-refractory prostate cancer (Ozpolat et al., 2003).

EndoTAG-1, paclitaxel embedded in a cationic liposome, is in Phase II trials for treatment of advanced triple-negative breast cancer (Awada et al., 2014) and pancreatic cancer (Löhr et al., 2012). EndoTAG-1 showed a greater antivascular effect on tumor vasculature and selective affinity to the target tumor (Chang and Yeh 2012). Another form of paclitaxel, LEP-ETU, in Phase I/II trials (Zhang et al., 2005; Immordino et al., 2006) presented a greater stability and reduced cardiotoxicity by using of cholesterol and cardiolipin in the liposomal formulation (Chang and Yeh 2012).

SPI-77, the first liposomes to deliver cisplatin, showed increased cisplatin tumor accumulation in preclinical models but limited clinical efficacy in a Phase II clinical trial of advanced non-small-cell lung cancer (White et al., 2006). Similarly, liposomal annamycin in Phase II has demonstrated no detectable antitumor activity in the treatment of Dox-resistant breast cancer (Booser et al., 2002).

Furthermore, the use of liposomes to deliver antigens derived from the influenza virus (Inflexal® V) or hepatitis A (Epaxal®), by intramuscular administration, has been approved. Inflexal V and Epaxal are liposomal vaccines. It was demonstrated that when viral membrane proteins or peptide antigens are incorporated into liposomes, cell-mediated and humoral immune response are potentiated (Stegmann et al., 1987; Glück et al., 1992). The oral administration of liposomal vaccines has also been considered, however, due to the potential for liposome breakdown following exposure to bile salts, injections remain the best route of administration for therapeutic peptides (Shaji and Patole 2008).

Drug	Disease	Status	Type of liposomal-based delivery system	Source(s)
Paclitaxel LEP-ETU	Advanced triple-negative breast cancer	Phase I/II	siRNA	Zhang et al., 2005; Immordino et al., 2006
siRNA	Ovarian cancer	Phase I	DOPC neutral liposomes	Mangala et al., 2009
Paclitaxel EndoTAG-1	Advanced triple-negative breast cancer	Phase II	Cationic	Chang and Yeh, 2012; Awada et al., 2014
Paclitaxel EndoTAG-1	Pancreatic cancer	Phase II	Cationic	Löhr et al., 2012
Mitoxantrone LEM-ETU	Acute myeloid leukemia, multiple sclerosis, and prostate cancer	Phase I	Cationic	Immordino et al., 2006; Chang and Yeh, 2012
Verteporfin	Molecular degeneration	FDA Approved in 2000	Cationic	Chang and Yeh, 2012; Allen and Cullis, 2013; Gross et al., 2013
Amikacin	Lung infection	Phase II/III	Conventional	Chang and Yeh, 2012; Clancy et al., 2013; Olivier et al., 2014
Vincristine	Non-Hodgkin lymphoma	FDA Approved in 2012	Conventional	Allen and Cullis, 2013; Wang et al., 2015
Tretinoin	Acute promyelocytic leukemia and hormone-refractory prostate cancer	Phase II	Conventional	Ozpolat et al., 2003; Immordino et al., 2006
Irinotecan SN-38	Metastatic colorectal cancer	Phase I/II	Conventional	Zhang et al., 2004; Suenaga et al., 2015
Annamycin	Acute lymphoblastic leukemia	Phase I/II	Conventional	Wetzler et al., 2013
Amphotericin B	Anti-fungal prophylaxis	FDA approved in 1997	Conventional	Chandrasekar, 2008; Allen and Cullis, 2013
Daunorubicin	Leukemia and solid tumors	FDA Approved in 1996	Conventional	Chang and Yeh, 2012; Allen and Cullis, 2013
Cytarabine or cytosine arabinoside	Neoplastic meningitis and lymphomatous meningitis	FDA Approved	Conventional	Chang and Yeh, 2012; Jahn et al., 2015
Morphine sulfate	Pain Management	FDA Approved in 2004	Conventional	Chang and Yeh, 2012; Allen and Cullis, 2013
Lurtotecan	Ovarian cancer, head, and neck cancer	Phase I/II	Conventional	Dark et al., 2005; Chang and Yeh, 2012
Vinorelbine	Newly diagnosed or relapsed solid tumors	Phase I	Conventional	Allen and Cullis, 2013
Topotecan	Advanced solid tumors	Phase 1/II	Conventional	Seiden et al., 2004; Allen and Cullis, 2013
Nystatin	Fungal Infections	Phase I/II	Conventional	Offner et al., 2004
Doxorubicin	Leukemia, breast cancer, bone cancer, lung cancer, brain cancer	FDA Approved in 1995	PEGylated	Ning et al., 2007
Doxorubicin and bortezomib	Relapsed or refractory multiple myeloma	FDA Approved in 2007	PEGylated	Ning et al., 2007
Thermosensitive doxorubicin	Liver tumors	Phase III	PEGylated	Yarmolenko et al., 2010
Thermosensitive doxorubicin	Chest wall recurrences of breast cancer	Phase I	PEGylated	Yarmolenko et al., 2010
Irinotecan	Advanced refractory solid tumors and colorectal cancer	Phase I	PEGylated	Chang et al., 2015
Camptothecin analog	Ovarian cancer	Phase I	PEGylated	Zamboni et al., 2009

Table 1: Liposomes on market and in clinical trials. Sercombe et al., 2015. *Front Pharmacol.* 6:286

3.5 Targeted liposomes for brain drug delivery

The liposomes, due their characteristics, are attractive transport systems to deliver drugs into CNS. The modification of liposome surface is most used strategy to direct therapeutic molecules to brain. Several studies have demonstrated that the conjugation with antibodies makes liposomes more recognizable by brain endothelial cells (Schmidt et al., 2003). PEGylated liposomes conjugated with monoclonal antibodies to glia, transferrin receptors (OX-26), lactoferrin receptors (Huang et al., 2013), low-density lipoprotein (LDL) receptors (Pinzón-Daza et al., 2012), or insulin receptors (Liu et al., 2008) are investigated to deliver drugs to the brain. Moreover, the conjugation of liposomes with diphtheria toxin receptor (Heparin binding [HB]-EGF) also represents a promising drug delivery system to cross the BBB (Gaillard et al., 2005). Different liposomes were considered efficient for therapy of brain tumors by remarkably increasing the delivery of antitumor drugs (O' Donnell et al., 2006). PEGylated liposomes

containing doxorubicin, in clinical trials, have shown effective for treatment of primary and metastatic brain tumors (Hau et al., 2004). For example, irinotecan encapsulated in nanoliposomes is significantly delivered to the brain, showing elevated antitumor efficacy (Krauze et al., 2007). Stearylamine-containing liposomes with covalently bounded transferrin on their surface increase the transport of 5-fluorouracil to the brain (17-fold) (Soni et al., 2008). Immunoliposomes labeled with OX-26 monoclonal antibody were evaluated efficient systems to deliver exogenous genetic material to the brain (Shi et al., 2001). Furthermore, the OX-26-coniugated PEGylated-liposomes are able to deliver a tyrosine hydrolase expression plasmid thus normalize tyrosine hydrolase activity in the striatum of adult rats Parkinson's disease model (Zhang et al., 2003).

Different studies have demonstrated that several substances encapsulated in liposomal form present more advantages compared to their "naked" forms. Among these, the nerve growth factor (NGF), a peptide with beneficial trophic effects on damaged neurons but with a low capacity to pass across the BBB. When entrapped into sterically-stabilized liposomes (SSLs), NGF shows better penetration through the BBB with a peak concentration in the brain within 30 minutes of intravenous administration (Xie et al., 2005). Liposomes loaded with galanthamine, an agent used for treatment of Alzheimer's disease, administered intranasally demonstrated a significant increase in efficacy in comparison with galanthamine delivered intranasally or orally (Li et al., 2012).

Recently, asialoerythropoietin (AEPO) loaded into PEGylated liposomes were investigated. AEPO is a metabolite of erythropoietin with brain cytoprotective activity. In a model of transient cerebral ischemia, if given at an early stage of reperfusion, AEPO-loaded PEGylated liposomes (AEPO-PL) accumulate rapidly in the brain, thus limiting the extent of damage. In terms of efficacy, AEPO-PL showed a 70% reduction of the infarct volume compared to a 30% reduction of infarct volume in animals treated with naked AEPO (Ishii et al., 2012), probably for longer retention of AEPO-PL in the infarct region compared to the short half-life of its naked compound.

Citicoline-loaded PEGylated liposomes (Citi-PL) also were investigated to evaluate the progression of infarct size in an animal model of stroke via serial magnetic resonance imaging. Animals treated with Citi-PL for 7 days showed a significant infarct size reduction in comparison with animals treated with citicoline free (Ramos-Cabrer et al., 2011). PEGylated liposomes conjugated to T7, a peptide that targeted to transferrin receptor, and loaded ZL006, a novel neuroprotectant, were investigated to treat ischemic stroke. T7 modification showed increased liposomes uptake by the brain capillary endothelial cells (BCECs) *in vitro* and significantly enhanced the transport of liposomes across the BBB *in vivo*. The pharmacodynamic studies suggested that these liposomes reduce infarct volume and ameliorate neurological deficit compared with unmodified liposomes or free ZL006 (Wang et al., 2015).

Furthermore, cell-penetrating peptides (CPPs) can be used to overcome limitations of brain transport. CPPs are peptides that readily penetrate through the cell membrane without causing damage to the membrane (Zhao et al., 2007). The CCPs mechanism of penetration is not fully known; probably, due to their structural peculiarities, they are able to "crawl" through the cell membrane (Banks et al., 2005). Among the CPPs TAT is the most known. It is a trans-activating HIV type 1 protein essential for virus replication and contains a basic domain that includes six arginine residues and two lysine residues. The cationic charges of TAT residues facilitate its interaction with the negatively charged BBB, thereby permitting the penetration through the BBB and accumulation in the CNS (Banks et al., 2005; Strazza et al., 2011; Toborek M et al., 2005). *In vivo* experiments have demonstrated that, PEGylated liposomes conjugated with TAT, penetrate the CNS and deliver doxorubicin to the brain in rats with glioma (Spuch and Navarro 2011; Qin et al., 2011).

Recently dual-targeting liposomal systems were also investigated. Daunorubicin, an anticancer agent, was tested loaded into liposomes and subsequently modified via conjugation with p-aminophenyl-α-d-manno-pyranoside (MAN) and transferrin (TF). MAN was conjugated to liposomes to facilitate the passage through the BBB, whereas TF to facilitate liposomes transfer into tumor tissues. In brain endothelial cells, it was found that the concentration of liposomes conjugated with both MAN and TF in the cells was the highest in comparison to free daunorubicin, and liposomes conjugated with MAN or TF. Moreover, uptake into C6 glioma cells and antiproliferative effect was also observed to be highest with liposomes dual-functionalized. These cellular level effects were translated into a significantly longer survival rate in animals with a brain tumor that was treated with liposomes conjugated with both MAN and TF (Ying et al., 2010).

Dual-targeting liposomal system modified with TAT and T7 and loaded with doxorubicin were evaluated *in vitro* and *in vivo*. The specific ligand T7 was used to target BBB and brain glioma tumor and the nonspecific ligand TAT to enhance the effect of passing through BBB and elevate the penetration into the tumor. The results *in vitro* showed that dual-targeting liposomes higher cellular uptake in the endothelial cells of BBB and penetrate more into C6 tumor spheroid compared with doxorubicin free and single-targeting liposomes. *In vivo* pharmacokinetic studies showed higher accumulation of doxorubicin in the brain of T7-TAT-liposomes compared all other experimental classes (Zong et al., 2014).

Finally, theranostic liposomes are exploring also for brain drug delivery. Quantum dots and apomorphine incorporated theranostic liposomes have been proposed to eliminate uptake by the liver and to enhance brain targeting (Wen et al., 2012).

4. gH625 PEPTIDE FOR DELIVERY ACROSS THE BBB

In the past few decades several peptides have been identified as drugs or used in drug design and pharmaceutical delivery. In fact, about 10% of the entire drug market is represented by peptide based drugs (Craik et al., 2013; Vlieghe et al., 2010). Among the various peptides those with high propensity to bind to lipid membranes result particularly interesting for therapeutic applications. Membranotropic viral peptides, due to their features and their way of interaction with membrane bilayers, can be used to promote the delivery of several cargos across the cell membrane and likely also across the blood-brain barrier. The peptide gH625 is a viral membranotropic peptide extensively used for many applications and among them as drug delivery system. The exact molecular mechanism with which gH625 enter in the cell remains to be established, but this peptide appears to be a promising tool to deliver several cargoes with intact bioactivity into different tissues or organs (Galdiero et al., 2014).

4.1 Membranotropic peptides

Recently, short cationic and/or amphipathic peptides, known as cell-penetrating peptides (CPPs), have been used to mediate the drug delivery (Drin et al., 2003; Farkhani et al., 2014; Wang et al., 2014; Capolovici et al., 2014). CPPs, short and usually basic amino acid rich peptides, are able to transport several types of macromolecules across the membrane bilayer *in vitro* and *in vivo* including small molecules, proteins, DNA/RNA, liposomes, peptides, and other supramolecular aggregates (Heitz et al., 2009; Vivès et al., 1997; Angeles-Boza et al., 2010). Although the uptake mechanism of CPPs is still debated, it seems to involve mainly the endocytic pathway. However, direct uptake by energy independent pathways have also been reported for specific CPPs (Conner and Schmid 2004; Wadia et al., 2004; Kaplan et al., 2005; Lundberg and Langel 2003; Thorén et al., 2000; Lonn and Dowdy 2015). On the basis of their hydrophilicity and hydrophobicity and thus on their different interaction with the membrane bilayer, CPPs are classified in cationic CPPs, membranotropic and amphipathic peptides (Falanga et al., 2015). Cationic CPPs, as the HIV-1 protein TAT, are characterized by a high content of arginine, lysine and histidine residues. The arginine guanidine head group forms hydrogen bonds with the negatively charged phosphates and sulphates on the cell membrane thus mediating the internalization in physiological pH conditions (Lonn and Dowdy 2015). Membranotropic and amphipathic peptides are constituted by hydrophobic amino acids and present a low net charge. Amphipathicity is a primary feature of these peptides and plays a key role in their interaction with the membrane bilayer and in their mechanism of internalization (Harris et al., 2000). Amphipathic CPPs present lipophilic and hydrophilic blocks or a lipophilic and hydrophobic face involved in the peptide translocation across the cell membrane (Bolhassani 2011). These peptides can be classified in primary amphipathic CPPs, secondary

amphipathic α-helical CPPs, β-sheet amphipathic CPPs, and proline-rich amphipathic CPPs (Milletti 2012).

Membranotropic CPPs include viral fusion peptides (Galdiero et al., 2014). They present an unusually high content of alanine and glycine residues and often also prolines and a high content of aromatic residues. The high content of Ala/Gly permits the intrinsic conformational flexibility typical of membrane interacting peptides. The aromatic residues form favorable interactions with phospholipid groups on the membrane interface and thus promote the insertion of peptide into bilayer (Yau et al., 1998). Their high propensity to bind to lipid membranes is due to their simultaneous hydrophobic and amphipathic nature. Moreover, their cellular uptake strongly depends by the secondary structure adopted after interaction with cellular plasma membrane (Harris et al., 2000). These peptides can be amphipathic in the primary structure presenting a sequential assembly of hydrophobic and hydrophilic domains separated by a spacer, or in the secondary structure with hydrophobic and hydrophilic residues located on opposite sides. Secondary amphipathic peptides present often large and aromatic residues on one face and small residues such as Ala/Gly on the other face, thus enhancing the interaction and insertion into membrane bilayer. Conformational polymorphism also plays a key role in the uptake mechanism; in fact, the ability to shift from random to α or β conformations following membrane interaction is a peculiar feature of this class of peptides (Joanne et al., 2009). In recent years, various structural models of interaction between peptides and membranes have described. Among these are membrane permeabilization through the formation of stable pores (such as barrel-stave and toroidal pore models) or micellization on a detergent-like way (carpet model) (Teixeira et al., 2012). In the barrel-stave model, peptide helices form well-defined and stable bundles used as a pore. In the toroidal-pore model, the lipids create a pore covered with peptides different orientations; hence the pores are less stable. In the carpet model, the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet (Brogden 2005). The strong affinity of membranotropic CPPs for the membrane bilayer is based on hydrophobic interactions. They self-associate at the interface between the membrane and the aqueous compartments, which could be relevant for direct translocation, and penetrate deeper into the hydrophobic core compared to cationic CPPs without span the bilayer in a pore-like manner. In addition, direct translocation can be achieved also via transient pore formation or carpet-like perturbations. Thus, direct translocation involves stable or transient destabilization of the membrane bilayer associated with folding of the peptide in the lipid membrane (Falanga et al., 2015). Therefore, membranotropic CPPs are able to efficiently cross biological membranes, promote lipid-membrane reorganizing processes (fusion or pore formation) and determine a local and temporary membrane destabilization with subsequent reorganization (Galdiero et al., 2012; Falanga et al., 2009). Some studies have demonstrated that the uptake mechanism may also depend on the CPP concentration. For cationic CPP the uptake occurs by endocytosis at low peptide concentrations but can switch to

direct uptake above a certain threshold (Fonseca et al., 2009). Whereas, for membranotropic CPPs direct penetration likely occurs at both high and low CPP concentrations. Although these concentrations change for different CPP, cell lines, type of cargo and mainly the mechanism of interaction with the membrane bilayer. In particular, when CPP transport a cargo inside cells the efficiency of uptake depend from several factors such as differences in the properties of the CPP (charge, hydrophobicity, length, secondary structure), nature of cargo (size, charge, type), cells lines and CPP and cargo concentrations. Due to these factors different internalization pathways may act simultaneously. However, cationic CPPs deliver a cargo essentially by endocytosis. By this way, the cargo may be degraded into lysosomal compartment without explicate its biological effects. Whereas, membranotropic CPPs uptake mainly occurs by direct penetration of the plasma membrane and, consequently, the cargo is immediate bioavailable (Galdiero et al., 2014; Guarnieri et al., 2013). Due to their peculiar properties of uptake, these peptides could reveal an interesting tool to deliver drug across the cell membrane.

4.1.1 Membranotropic peptides as delivery tools

Membranotropic CPPs represent particularly attractive tools for drug delivery. Their direct translocation across membranes could be immediately available in the cytosol the cargo transported, thus avoiding the risk of endosomal entrapment and degradation (Galdiero et al., 2013). Several membranotropic CPPs are already used to deliver a cargo in the cells. Among these is CADY, a 20 residue peptide, containing both aromatic tryptophan residues and cationic residues. It assumes an amphipathic helical conformation in the bilayer, involved in the interactions with the cell membrane and in the mechanism of cell penetration. CADY has been used to improve cellular uptake of siRNA and its internalization is rarely influenced by inhibitors of the endosomal pathway (Konate et al., 2010).

MPG, a 27 amino acid peptide, is composed of the hydrophobic amino acids of the HIV-1 fusion peptide associated to a hydrophilic domain derived from the Nuclear Localization Sequence (NLS) of Simian virus 40 (SV40) large T antigen (Morris et al., 1997; 1999). The glycine-rich HIV fusion peptide is essential for membrane fusion activity while the NLS of the SV40 large T antigen improves the nuclear addressing of the peptide (Gallaher 1987; Kalderon and Smith 1984). *In vitro* studies have demonstrated that MPG can deliver both siRNA and DNA after just 1 h (Morris et al., 1999). MPG cellular uptake occurs mainly by cell translocation mechanism that is independent of endosomal pathway. Its insertion into the membrane and its initiation of translocation involve membrane disorganization and folding into β-structures within the membrane bilayer without any associated leakage or toxicity.

Another membranotropic peptide is Pep-1, partially derived from MPG, that conserves the C-terminal hydrophilic domain corresponding to the NLS of SV40 large T antigen and its hydrophobic region is a tryptophan-rich sequence derived from the HIV-1 reverse transcriptase

(Morris et al., 2001; Deshayes et al., 2004). Pep-1 may cross the membrane bilayer by a physical mediated mechanism, promoted by the transmembrane potential, without pore formation. It has been demonstrated that this peptides is able to efficiently deliver various cargoes into different cell lines in a fully active form (Morris et al., 2001; Morris et al., 2008; Henriques and Castanho 2008).

Herpes Simplex Virus Type 1 (HSV-1) Protein VP22 (Elliott GD and Meredith DM 1992), peptide involved in the traffic between cells (Sciortino et al., 2001), is able to deliver DNA and RNA oligonucleotides into cells (Normand et al., 2001; Sciortino et al., 2002).

C105Y, a synthetic peptide based on the amino acid sequence of a1-antitrypsin, is able to enter very rapidly in cytoplasm, nucleus, and nucleolus of live cells. Its uptake and internalization not involve known endocytic pathways. *In vitro* and *in vivo* studies, have been demonstrated that C105Y, attached to polyK to condense plasmid DNA, can 100-fold increase gene expression compared to polyK-DNA complexes without C105Y (Rhee and Davis 2006).

VT5, a synthetic water soluble amphipathic 26-mer β-sheet peptide, enhances internalization into endothelial cells. Although its mechanism of uptake is not yet known, it seems to be energy, pH and temperature dependent (Oehlke et al., 1997). Finally, transportan and TP10, synthetic peptides derived from the N-terminal domain of the neuropeptide galanin linked through a lysine residue to mastoparan, translocate across the membrane bilayer and deliver different cargoes (Pooga et al., 2001).

4.2 gH625: membranotropic peptide derived from *Herpes simplex virus* type I

Among membranotropic peptides, those derived from enveloped virus glycoproteins are particularly interesting due to their wide applicability. However, they are also more complicated to understand because they are involved both in virus fusion and entry. The entry of viruses into the cells occurs by several membrane reorganization processes similar to the ones involved in delivery across cellular membranes. The high content of aromatic residues determines the insertion of these peptides into only one leaflet of the bilayer (Larsson and Kasson 2013), thus permitting the formation of bulges that stick out of the membrane and improving the contacts between fusing bilayers (Kozlov et al., 2010).

gH625 is a viral membranotropic peptide derived from glycoprotein H of Herpes simplex virus type I (HSV-1) (Galdiero et al., 2015). *Herpes simplex virus* is an important human pathogen able to enter host cells by fusion of the viral envelope with the plasma membrane or an endosomal membrane. Its entry pathway involves multiple viral glycoproteins and cellular receptors in a cascade of molecular interactions (Delboy et al., 2006; Roller et al., 2008; Arii et al., 2009; Milne et al., 2005). The envelope glycoproteins gH/gL, gB and gD are all essential for the entry process, inducing the fusion of cellular membranes in a virus-free system (Connolly et al., 2011; Turner et al., 1998). gH/gL and gB induce the initial lipid destabilization that ends in

fusion (Farnsworth et al., 2007). Both gB and gH contain several membranotropic sequences, which are involved in the local destabilization of the membrane bilayer, thus determining the fusion of the viral and host cell membranes (Falanga et al., 2012; Galdiero et al., 2005, 2008, 2012; Akkarawongsa et al., 2009).

gH625, characterized by Galdiero S and colleagues in 2005 (Galdiero et al., 2005), is the HSV-1 peptide with the highest fusion capability **(Figure 11)**. The 20-residue peptide gH625, from aa 625 to aa 644, is a membrane-perturbing domain, containing key residues to interact and destabilize biological membranes (Almeida 2014; Vivès et al., 1997; Morris et al., 1997). It is rich in hydrophobic residues including glycines, leucines, alanines, and aromatic residues such as tryptophan and tyrosines, located preferentially at the membrane interface. This hydrophobic domain is crucial for insertion of peptide into the membrane and is involved in membrane perturbation in the early stages of viral fusion. Although the exact molecular mechanism of gH625 entry remains to be established, gH625 has been shown to interact strongly with and penetrate the lipid-phase spontaneously and insert into membranes (Galdiero et al., 2010). The peptide-lipid interactions are initiated by the arginine residue located at the C-terminus (Falanga et al., 2011). Moreover, the presence of histidine residue at the N-terminus of the native sequence facilities the initial interactions with the membrane and the oligomerization process, by strongly increasing the fusion activity (Galdiero et al., 2005; 2008; 2010; 2012). The hydrophobic and amphipathic profile of gH625 permit the interaction with membrane lipids and the formation of a transient helical structure that temporarily affects membrane organization, by facilitating insertion into the membrane and translocation (Galdiero et al., 2015; Falanga et al., 2011). The amphipathic helices would allow enter the membrane and trigger local fusion of the membrane leaflets, transient pore formation, cracks and membrane fusion (Bareford and Swaan 2007; Galdiero et al., 2014; Almeida et al., 2014; Heitz et al., 2009). The gH625 ability to penetrate deep into the membrane bilayer, without significant bilayer perturbations, may help to explain its ability to perform several different roles (Galdiero et al., 2014).

Some studies have shown a significant viral inhibitory effect of gH625 related to its ability to partition into membranes and aggregate within them (Galdiero et al., 2008).

The substitution of Leu627 with a polar residue, such as serine, substantially reduces the inhibitory activity of peptide (Galdiero et al., 2008). It has been demonstrated that poly(amide)-based dendrimers functionalized with gH625 may inhibit both HSV-1 and HSV-2 at a very early stage of the entry process, likely by interaction with the viral envelope glycoproteins (Tarallo et al., 2013). These modified dendrimers increase almost six fold the antiviral activity for HSV-1 and two fold for HSV-2 in comparison to the dendrimer itself, and more than 100-fold increase in the activity of the unsupported peptide. This antiviral activity was not associated to cytotoxicity for functionalized dendrimers, while toxicity was observed for unfunctionalized dendrimer, showing another advantage of the peptide (Tarallo et al., 2013). However, peptidodendrimer and the dendrimer are not able were to interfere with viral replication at a

virus post-entry step. Dendrimer scaffold modified with membranotropic peptides are a promising strategy to design new antiviral drugs that, coupling the intrinsic anti-viral properties of the dendrimer with the activity of membranotropic peptides, may deliver therapeutic agents directly to target (Tarallo et al., 2013). Furthermore, many studies have been demonstrated that gH625 is able to deliver cargoes in several *in vitro* cell lines (Galdiero et al., 2013, 2014; Vitiello et al., 2011).

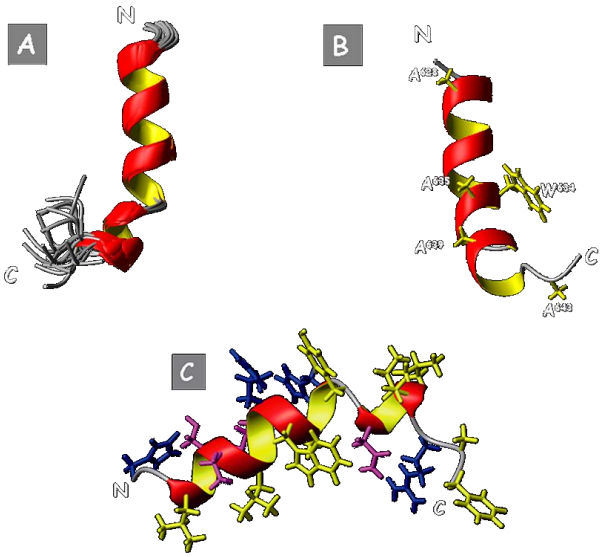

Figure 11: Structures of gH625. A) Backbone atoms superposition (residues 627−640) of the 20 energy-minimized structures of gH625−644; **B)** Representative conformer of gH625-644 NMR structure; **C)** The amphiphilic character of gH625−644. Blue: charged residue side chains; violet: polar residues; yellow: hydrophobic residues. Galdiero et al., 2012. *Biochemistry*, 51 (14), 3121–3128

4.2.1 gH625 as drug delivery tool

gH625, compared to TAT peptide which mainly exploits the endocytic pathway, crosses membrane bilayers mainly through a translocation mechanism. Moreover, a one amino acid shorter version of this fusogenic peptide improves the endosomal release of DNA/Lipofectamine lipoplexes and transgene expression up to 30-fold in human cell lines (Tu and Kim 2008). Several studies have been demonstrated that gH625 directly translocate across the membrane bilayer and to transport into the cytosol several cargoes such as Quantum Dots

(QDs) (Falanga et al., 2011), liposomes (Tarallo et al., 2011), polystirene nanoparticles (NPs) (Guarnieri et al., 2013), dendrimers (Carberry et al., 2012), and proteins (Smaldone et al., 2013). QDs are fluorescent probes particularly interesting for their many applications in molecular, cellular and in *vivo* imaging (Pinaud et al., 2010). Various studies have demonstrated that the functionalization of QDs with the TAT peptide or other positively charged CPPs enhance the cell penetration, and the main route of uptake is by endosomal pathway; therefore, escape from the endosomal compartment is necessary (Medintz et al., 2008; Lee et al., 2010; Delehanty et al., 2011) **(Figure 12)**. QDs functionalized with gH625 showed more effectively uptake than QDs functionalized with TAT, and their internalization only partially occurs by endocytic pathway. Moreover, gH625-QDs are present in the cytoplasm in a more punctuated form in comparison with TAT QDs (Falanga et al., 2011).

Dendrimers are perfectly branched macromolecules with a well-defined structure considered a very promising tool for drug delivery (Newkome et al., 2001; Lee et al., 2005). Dendrimers functionalized with gH625 are able to enter cells by a passive translocation mechanism, allowing the cargo to be released directly into the cytoplasm (Borchmann et al., 2015; Carberry et al., 2012; Falanga et al., 2014).

gH625 was also used to functionalize liposomes. Liposomal formulations have also attracted great attention due to their success as *in vivo* carriers of drugs (Cukierman and Khan 2010). The addition of ligands and cell penetrating peptides on the liposome surface is a strategy to enhance the antitumor efficacy of liposomal drugs. Liposomes functionalized with gH625 on their external surface and loaded with doxorubicin (DOXO) accumulate in the cytoplasm without entering the nucleus conversely than free Dox and Dox-loaded liposomes unfunctionalized. These results suggest that the functionalization of liposomes with gH625 could affect the uptake mechanism of liposomes, escaping the lysosome accumulation, allowing a more homogeneous intracytoplasmic distribution, and modulating the Dox release (Tarallo et al., 2011).

Moreover, recently, it was evaluated the effects of increasing concentrations of liposomes encapsulating Doxo decorated with gH625 (LipoDoxo-gH625) on growth inhibition of wild type (A549) and doxorubicin-resistant (A549 Dx) human lung adenocarcinoma (Perillo et al., 2015). The results showed a higher growth inhibition induced by LipoDoxo-gH625 than that caused by liposomes encapsulating Doxo undecorated, in both cell lines. The data on cell growth inhibition were paralleled by a higher oxidative stress and an increased uptake of Doxo induced by LipoDoxo-gH625 compared to LipoDoxo, above all in A549 Dx cells. Therefore, the functionalization of liposomes with gH625 may overcome doxorubicin resistance in lung adenocarcinoma cell lines (Perillo et al., 2015).

Hence, all these results taken together show that gH625 represent a promising delivery tool.

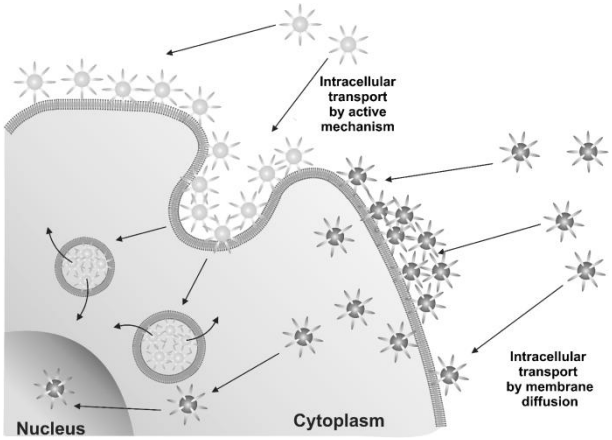

Figure 12: Intracellular delivery: endocytic mechanisms compared to translocation. Falanga et al., 2013, *Journal of Nanophotonics* 071599-1

4.2.2 gH625: *in vitro* delivery across the blood–brain barrier

Many therapeutic molecules are not able to enter in the brain, due to the presence of BBB (Lu et al., 2014). Targeted delivery of therapeutic molecules to the brain seems to be one of the most promising non-invasive strategies to overcome the BBB (Mahajan et al., 2012; Jain 2012; Orive et al., 2010; Qiao 2012).

Nanocarriers functionalized with gH625 are evaluated using an *in vitro* BBB model based on bEnd3, an immortalized mouse cerebral endothelial cell line. 100-nm polystyrene nanoparticles (NPs) decorated on their surface with gH625 show greater uptake by brain endothelial cells than NPs without the peptide, and their intracytoplasmatic motion was mainly characterized by a random-walk behavior indicative of a passive mechanism of internalization. Interestingly, the presence of gH625 decreased NP intracellular accumulation as large aggregates and enhanced the NP passage of the BBB. Therefore, gH625 likely may change the NPs fate and provides a good strategy to design promising carriers to deliver drugs across the BBB (Guarnieri et al., 2013).

Furthermore, a recent study has evaluated the ability of aminated polystyrene nanoparticles functionalized with gH625 (NPs-gH625) to cross the brain endothelial cell membrane (bEnd3) and their nanotoxicological impact. A significant number of unfunctionalized NPs (blank-NPs) were observed associated with lysosomes; after 24h the degree of lysosomal accumulation was around 80%. gH625-NPs only slightly co-localized with lysosomes, and their degree of lysosomal accumulation did not increase with time. Moreover, it has been shown that decoration

of NPs with gH625 significantly reduce cytotoxicity compared to blank-NPs, likely due to the ability of gH625 to avoid accumulation into lysosomes (Guarnieri et al., 2015).

Therefore, gH625 provides several advantages. Its mechanism of internalization involves the endocytic pathway only partially; it facilitates the escape from endocytic vesicles; finally, it shows lower toxicity due to the absence of accumulation in lysosomes. However, is not fully granted that nanosystems designed and tested *in vitro* are able to properly work *in vivo*. To address this issue, in vivo studies are necessary, thus validating design strategies and facilitating optimization and further functionalization.

5. NEUROPROTECTIVE EFFECTS OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) IN NEUROLOGICAL DISORDERS

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide widely distributed in the central and peripheral nervous systems which acts as a neuromodulator, neurotransmitter, and neurotrophic factor (Lee and Seo 2014). Several studies have shown potent neurotrophic and neuroprotective effects of PACAP *in vitro* and *in vivo* and it has been proposed as a treatment for CNS injuries, stroke, and neurodegenerative diseases. By intravenous injection PACAP is transported across the BBB by a saturable transport system and shows uptake highest in the hypothalamus and hippocampus (Nonaka et al., 2002). Use of PACAP as intravenous therapeutic agent presents some problems such as its rapid degradation in the blood and low bioavailability. Moreover, the presence of a brain-to-blood efflux transporter on the BBB limits its amount in the brain (Meredith et al., 2015). Based on published data, PACAP may become useful a therapeutic agent in many neurological disorders characterized by neurodegeneration, such as cerebral ischemia, traumatic brain injury, Parkinson's disease, and Alzheimer's disease. However, it needs to be developed strategies to improve and target the delivery of PACAP to the CNS.

5.1 Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP is a polypeptide encoded by the ADCYAP1 gene in human, isolated from ovine hypothalamus and named after its ability to stimulate 3',5'cyclic adenosine monophosphate (cAMP) formation in rat anterior pituitary cells (Miyata et al., 1989). It is expressed in the central nervous system and in different other organs such as retina, immune cells, kidney, gastrointestinal tract, reproductive tissues, etc. PACAP exists in two forms: PACAP27 and PACAP38 with 27- and a 38-amino acid respectively (Miyata et al., 1990), processed from a prohormone precursor (Läuffer J et al., 1999). Both forms of PACAP have been shown to stimulate adenylate cyclase release in several cell types including pituitary cells, pancreatic acinar cells, neurons, and astrocytes. The predominant form, PACAP38, is found primarily in the hypothalamus, hippocampus, posterior pituitary, cerebral cortex, testes and adrenals (Miyata et al., 1989; Kimball et al., 1996). Due to its amino acid composition, PACAP belongs to secretin/glucagon/vasoactive intestinal polypeptide (VIP) superfamily. Three G protein coupled seven transmembrane receptors (GPCRs) have been cloned and identified as major PACAP receptors, PAC1, VPAC1, and VPAC2. PAC1 is a PACAP-selective receptor, whereas $VPAC_1$ and $VPAC_2$ respond to both VIP and PACAP with high affinity (**Figure 13**) (Journot et al., 1994; Pantaloni et al., 1996; Hosoya et al., 1993). All these receptors are coupled to adenylate cyclases (ACs) and increase intracellular concentrations of cAMP. However, other pathways are often activated or inhibited in some cells in parallel or downstream of cAMP, including pathways involving exchange proteins activated by cAMP (Ster et al., 2007), PLC

(Spengler et al., 1993), NO (Murthy et al., 1993), src (Koh, 1991), phosphatidylinositol 3-kinase (Straub and Sharp, 1996), MAPK (Barrie et al., 1997; Villalba et al., 1997; Lelievre et al., 1998), Jak/STAT and NF-kB (Delgado and Ganea, 1999; 2000). PAC1 receptor can also be coupled to phospholipase Cβ (PLCβ) and produce inositol phosphate by activating IP3 receptor-mediated Ca^{2+} mobilization (Harmar 2001). PACAP is expressed throughout the central nervous system (CNS), such as in the hypothalamus, cerebellum, hippocampus, and substantia nigra (Arimura et al., 1994; Hannibal 2002), and in the peripheral nervous system (PNS) in the sensory neurons, and parasympathetic ganglionic neurons, sympathetic preganglionic neurons (Sundler et al., 1996). The exact localization of PACAP mRNA expression in the developing and adult rat brain and the presence of PACAP and PAC1 receptor mRNA are well known, suggesting its important function in these brain regions (Skoglosa et al., 1999; Watanabe et al., 2007). PACAP mRNA is expressed in alternating layers of the cerebral cortex (I, III, and V layer), to the CA1 and CA4 subregions of the hippocampus and the dentate gyrus, but it is not expressed in the CA2 or CA3 regions. PAC1 receptor mRNA is highly present in the dentate gyrus and less in other parts of the hippocampus and the cortex (Skoglosa et al., 1999). PACAP performs diverse roles in the nervous system such as neuromodulator and neurotrophic factor (Arimura et al., 1994; Somogyvari-Vigh et al., 2004; Botia et al., 2007; Ravni et al., 2006). In addition, several studies have demonstrated a neuroprotective effect of PACAP *in vitro* and *in vivo* in various models of ischemia, retinal degeneration and neurodegenerative diseases (Dejda et al., 2005; 2008; Brenneman 2007; Ohtaki et al., 2008; Bourgault et al., 2009; 2011; Atlasz et al., 2010; Nakamachi et al., 2011; Reglodi et al., 2011).

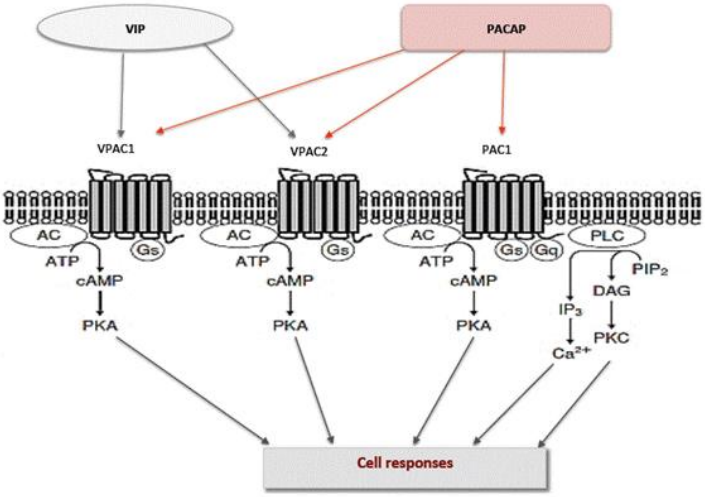

Figure 13: Schematic representation VIP/PACAP pathways. VIP or PACAP binds to the N-terminal domain of the VIP/PACAP receptor. The transduction signals occur via the adenylate cyclase or phospholipase C pathways or mobilizing intracellular calcium. **VIP:** Vasoactive Intestinal Peptide; **PAC1:** PACAP specific receptor 1; **VPAC1:** Receptor-1 for VIP; **VPAC2:** Receptor-2 for VIP; **AC:** Adenylyl Cyclase; **DAG:** Diacylglycerol; **IP3:** Inositol Trisphosphate; **PKA:** Protein-Kinase A; **PKC:** Protein- Kinase C; **cAMP:** Cyclic Adenosine Monophosphate; **ATP:** Adenosine Triphosphate; **PIP2:** Phosphatidylinositol Biphosphate; **PLC**: Phospholipase-C. Diané 2014, *J Metabolic Synd* 3:162

5.2 Neurotrophic and neuroprotective roles of PACAP

The wide distribution of PACAP in the nervous system indicates that the peptide has pleiotropic functions. PACAP performs diverse activities; it acts as a neurohormone, a neurotransmitter, and a neurotrophic factor. Different studies have also shown a neuroprotective effect of PACAP in several neurodegenerative diseases (Lee and Seo 2014). In the developing CNS it behaves as a neurotrophic factor, promoting cell survival and differentiation in different cells such as cerebellar granule cells, dorsal root ganglion cells, and cortical neuroblast (Gonzalez et al., 1997; Vaudry et al., 2000; Lioudyno et al., 1998). Moreover, PACAP inhibits apoptotic cell death and promotes survival and regeneration under several pathological conditions in mature brain. It has been demonstrated that in cultured cells, PACAP promotes the survival of rat cortical neurons against glutamate-induced toxicity (Morio et al., 1996) and of dopaminergic neurons against 6-hydroxydopamine-induced neurotoxicity (Chen et al., 2006). PACAP also prevents serum and NGF withdrawal-induced cell death in differentiated PC12 cells and primary sympathetic neurons (Tanaka et al., 1997; Waschek 2002; 2013; May et al., 2010). Its

neurotrophic and neuroprotective effects are mediated by direct or indirect mechanisms (Dejda et al., 2005). These actions of PACAP may occur by activation of the cAMP-protein kinase A (PKA) (Tanaka et al., 1997; Shoge et al., 1999) or the mitogen activated protein kinase (MAPK) pathway (Pugh and Margiotta 2006; Villalba et al., 1997). A direct protective effect of PACAP on neurons involves often inhibition of caspase-3, an apoptotic enzyme (Vaudry et al., 2000). In rat cortical neurons, the neuroprotective action of PACAP occurs by induction of transcriptional target gene expression, such as BDNF (Frechilla et al., 2001). Furthermore, PACAP may inhibit the expression of proapoptotic factors, such as Bcl-2-associated X protein (Bax), and activates the phosphatidylinositol 3'-OH kinase (PI3K) pathway (Falluel-Morel et al., 2004; Bhave and Hoffman 2004) (**Figure 14**). PACAP can also indirectly perform neuroprotective actions by modulating glial cells to provide neurotrophic support and control of inflammatory responses (Delgado and Ganea 2003). PACAP induces astroglial cells to release interleukin-6 (IL-6) in ischemia *in vivo* to protect neurons (Gottschall et al., 1994; Ohtaki et al., 2006). The protective effects of PACAP have been shown in various models of neurodegenerative diseases such as models of Parkinson's disease (PD) and different *in vitro* and *in vivo* models of Alzheimer's disease (AD) (Reglodi et al., 2011). In PD, PACAP protects dopaminergic neurons against 6-OHDA-induced neurotoxicity (Takei et al., 1998), and cholinergic neurotransmission, balancing the dopamine-acetylcholine systems in the basal ganglia neuronal pathway (Wang et al., 2008). In AD, the peptide prevents tau cleavage (Metcalfe et al., 2012), improves learning in a mouse (Dogrukol-Ak et al., 2009), and protects against β-amyloid toxicity (Han P et al., 2014a, 2014b). Moreover, some studies have demonstrated the neuroprotective effects of PACAP in other acute and chronic brain injury models: PACAP showed strong neuroprotective action in traumatic brain injury by reducing axonal damage in both impact acceleration and fluid percussion models (Tamas et al., 2012). PACAP also improves both functional and morphological symptoms in a model of spinal cord injury (Tsuchida et al., 2014; Tsuchikawa et al., 2012). Recent studies have suggested that its neuroprotective effect is due to the antioxidant and anti-inflammatory/immunomodulatory actions (Hua et al., 2012, Mao et al., 2012; Miyamoto et al., 2014). Furthermore, PACAP has protection effect in inflammatory disorders of the CNS, such as in models of multiple sclerosis. The administration of PACAP suppresses a model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Kato et al., 2004). Studies in mice showed that the endogenous production of the peptide protects against multiple sclerosis, probably by modulating regulatory T cells (Tan et al., 2015 and Tan and Waschek 2011). The functions of PACAP in neuronal regeneration suggest that PACAP may also be a promising therapeutic agent in injuries of the nervous system (Lee EH and Seo SR 2014).

Use of PACAP as a therapeutic agent is possible because PACAP crosses the BBB at a modest degree and uptake is highest in the hypothalamus and hippocampus (Nonaka et al., 2002). PACAP-27 can enter in the brain by transmembrane diffusion, while PACAP-38 by a saturable transport system (Banks et al., 1993). However, use PACAP as therapeutic agent involves

different problems such as its rapid degradation in the blood and low bioavailability and, also the presence of a brain-to-blood efflux transporter on the BBB, which limits the amount of PACAP into the brain. The peptide transport system-6 (PTS-6) is responsible for the bidirectional transport of PACAP-38 and for the efflux of PACAP-27 (Dogrukol-Ak et al., 2009). Recently, it has been shown that intranasal administration of PACAP38 occurs rapidly with the highest levels seen in the striatum and occipital cortex (Nonaka et al., 2012) with an effect on CNS function. Moreover, daily intranasal administration of PACAP for 3 months in a genetic AD mouse model, APP [V717I] transgenic mice, altered amyloid precursor protein (APP) processing by increasing secretion of the neuroprotective sAPPα and improved cognition (Rat et al., 2011). PACAP has been used in a number of clinical trials to treat several disorders including migraines and depression (Clinical Trial NCT00944996) (Amin et al., 2014) and a metabolically stable PACAP derivative, acetyl-[Ala^{15}, Ala^{20}] PACAP38-propylamide, is being developed (Bourgault et al., 2008). Based on published data, PACAP may become an useful therapeutic agent in many neurological disorders characterized by neurodegeneration. However, to avoid the problems connected to therapeutic use of PACAP, it is necessary to develop new strategies to improve its delivery onto the brain.

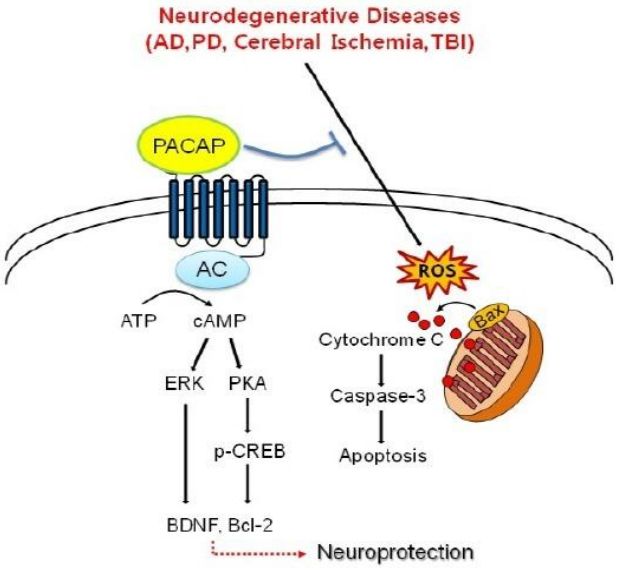

Figure 14. Neuroprotective pathway of PACAP in neurodegenerative diseases. By PAC1 receptors, PACAP activates adenylate cyclase (AC)-linked signal transduction pathway. PACAP triggers the anti-apoptotic transcriptional target gene expression and also inhibits apoptotic signaling responses, including ROS generation, mitochondrial Bax and cytochrome C release, and subsequence caspase-3 activation. Lee and Seo 2014, *BMB Rep*, 47(7): 369-375.

5.3 PACAP and neurodegenerative diseases

5.3.1 Cerebral ischemia

Cerebral ischemia or stroke is due to decreased blood flow to the brain which causes consequent decrease of oxygen and glucose. Global ischemia occurs for total loss of blood flow to the brain, while focal cerebral ischemia for local interruption due to cerebral artery occlusion (Dejda et al., 2011). PACAP showed significant neurotrophic and neuroprotective effects after stroke by preventing ischemic neuronal damage in transient global and focal cerebral ischemia (Banks et al., 1996). Intracerebroventricular or intravenous administration of PACAP in a model of transient global ischemia prevents the ischemic death of rat CA1 neurons, also until 1 day after the ischemic event (Uchida et al., 1996). Moreover, systemic administration of PACAP decreases infarct volume in a rat model of focal ischemia and ameliorates neurological defects if administrated 4 h after middle cerebral artery occlusion (MCAO), a mouse model of stroke (Reglodi et al., 2000). In addition, PACAP-deficient mice show more vulnerability following MCAO (Chen Y et al., 2006) with infarct volumes and neurological deficits greater than in the wild-type mice. PACAP acts on the mitochondrial apoptotic pathway to inhibit caspase-9 and subsequent caspase-3 activation as demonstrated by higher cytoplasmic cytochrome c levels and lower Bcl-2 expression in PACAP-deficient mice than wild-type mice (Ohtaki H et al., 2006). Studies on transcriptome alterations during ischemic insult in wild type and PACAP deficient mice suggest the possible involvement of Ier3, met enkephalin, substance P, and neurotensin expression in its neuroprotective effects (Chen et al., 2006). Furthermore, PACAP activates the DNA repair function of apurinic/apyrimidinic endonuclease 1 (APE1) (Stetler et al., 2010). PACAP may act on several periods of stroke to exercise neuroprotection. It may protect against glutamate-induced cytotoxicity and excitotoxic concentrations of glutamate typical of the acute period (Morio et al., 1996; Shintani et al., 2005); it may inhibit ROS-induced cell death in several cell types in the subacute periods (Vaudry et al., 2002; Horvath et al., 2010) and decrease the neuroinflammatory response and attenuates microglial activation characteristic of the chronic period (Armstrong et al., 2008; Suk et al., 2004).

5.3.2 Traumatic brain injury

Physical damage to the brain, known as traumatic brain injury (TBI), is a major responsible factor to death and chronic disability in individuals under the age of 45 years worldwide (Bruns and Hauser 2003; Werner and Engelhard 2007). TBI involves a complex neurodegenerative process, including many pathways (Raghupathi 2004). Several neurotrophic factors have been shown to be effective in cerebral ischemia and traumatic brain injury when given exogenously (Dietrich et al., 1996; Kawamata et al., 1997; Truettner et al., 1999; Leker and Shohami 2002). Although with different pathogenesis, cerebral ischemia and TBI share some common pathways

including excitotoxicity, nitric oxid production, ROS generation, elevated Ca^{2+} levels, and apoptosis (Leker and Shohami 2002; Povlishock 1992; Maxwell et al., 1997; Buki et al., 2000; Bramlett et al., 2004). Based on the neurotrophic and neuroprotective effect of PACAP in cerebral ischemia (Ohtaki et al., 2008; Uchida et al., 1996; Reglodi et al., 2000; 2002; Tamas et al., 2002), several studies have been performed to evaluate the effects of PACAP on different models of TBI. These studies have demonstrated that moderate TBI in rat brain induces changes in the mRNA expression of PACAP and the PAC1 receptor in the cortex and hippocampus (Skoglosa et al., 1999). After different central and peripheral nerve injuries, the upregulation of endogenous PACAP and its receptors show the important function of PACAP in the neuronal regeneration (Tamas et al., 2012). It promotes neural restoration by enhancing neurogenesis, angiogenesis, and neuroprotective effects in TBI (Johanson et al., 2011). PACAP treatment significantly reduces the diffusion of axonal injury and protects the cortocospinal tract in a rat model of TBI, induced by central fluid percussion (Kovesdi et al., 2008). Cerebroventricular microinjections of PACAP, in a weight-drop model of TBI, significantly improve motor and cognitive dysfunction, attenuate apoptosis, and decrease brain edema (Mao et al., 2012). Brain trauma, like other neuronal diseases, leads inflammatory response as a common pathological reaction (Ziebell and Morganti-Kossmann 2010). In case of TBI, the cerebral inflammatory response involves the activation of macrophages/microglia, neurons, and astrocytes, and increase the release of inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL1β) (Marklund et al., 2006). PACAP has shown immunomodulatory properties and inhibits production of TNF-α from microglia activated by lipopolysaccharide (LPS) *in vitro* (Fang et al., 2010; Kim et al., 2010). Administration of exogenous PACAP inhibits the secondary inflammatory response in microglia and neurons in TBI, by TLR4/MyD88/NF-κB pathway (Mao et al., 2012). Finally, PACAP inhibits the expression of IL-12, thereby suppressing T cell proliferation induced by TBI (Reglodi et al., 2011).

5.3.3 Alzheimer's disease

The development of Alzheimer's disease (AD) is characterized by deposition of amyloid β peptide (Aβ) (Hardy and Selkoe 2002). A group of enzymes called α-, β-, and γ-secretases, by proteolytic sequential cleavages of the amyloid precursor protein (APP), produce Aβ. A disintegrin and metalloproteinase-family enzyme (ADAM family) acts as α-secretase; β-site APP-cleaving enzyme 1 (BACE1) acts as a β-secretase. The γ-secretase is a complex of enzymes, composed of presenilin 1 or 2 (PS1 or PS2), nicastrin, anterior pharynx defective and presenilin enhancer 2 (LaFerla et al., 2007). When proteolytic cleavage of APP occurs by α-secretase, the formation of amyloidogenic peptides is precluded and soluble N-terminal APP fragments (sAPPα) with neurotrophic and neuroprotective properties are released. Some studies suggest that the neuroprotective action of PACAP in AD is mediated by stimulating α-secretase

activity. SK-N-MC neuroblastoma cells, which express endogenous PAC1 receptors, treated with PACAP have shown enhanced secretion of sAPPα than untreated cells. Furthermore, α-secretase cleavage of APP is strongly stimulated in HEK cells which overexpress functional PAC1 receptors (Kojro et al., 2006). PACAP also shows a potent neuroprotective effect in rat PC12 cell cultures over a long period at a very low concentration from Aβ-induced cytotoxicity (Onoue et al., 2002). Moreover, several studies have been demonstrated neuroprotective effect of PACAP also in *in vivo* models of AD. PACAP treatment enhances non-amyloidogenic pathway of APP processing and improves cognitive function in the brain of the AD transgenic mouse model, APP [V717I] (Rat et al., 2011). Significant downregulation of PACAP in several AD mice models and in the human AD temporal cortex supports its physiological relevance in AD (Wu et al., 2006). It is known that brain uptake of PACAP is limited by an efflux component, such as peptide transport system-6 (PTS-6). PACAP with antisense-PTS has been demonstrated to improve cognition, by inhibiting the peptide efflux pump, in SAMP8 AD mouse model (Dogrukol-Ak et al., 2009). Finally, there are different studies that evidence the involvement apolipoprotein (ApoE), a lipid carrier, in AD (Strittmatter and Roses 1995). Therefore, a mouse deficient in ApoE is a useful *in vivo* model to study development and degeneration (Plump et al., 1992). VIP, a PACAP family member, has showed protection from developmental retardation and memory deficits in ApoE-deficient mice (Gozes et al., 1997).

5.3.4 Parkinson's disease

In Parkinson's disease (PD) the damage or destruction of dopaminergic neurons in the substantia nigra (SN) causes by motor movement disorders (Jankovic 2008), which can be associated to cognitive and behavioral disturbances. It has been demonstrated that PACAP is able to protect PC12 cells from apoptosis induced by rotenone, which seems to induce PD by interrupting mitochondrial complex I activity leading to death of nigral neurons (Wang et al., 2005). The protective effect observed in these cells is linked to MAPK activation by PKA and involves caspase-3 inhibition (Wang et al., 2005). PACAP protects dopaminergic neurons against rotenone-, 6-OHDA-, and MPP^+-induced toxicity in cell culture (Takei et al., 1998; Chung et al., 2005). PACAP protects SH-SY5Y dopaminergic cells in salsolinol (SALS)-induced PD models (Brown et al., 2013). Recent studies on PD pathology show that several cellular and molecular events, such as microglia-mediated inflammation, oxidative stress, and apoptotic mechanisms, are likely involved in the neurodegenerative process (von Bohlen und Halbach et al., 2004). The neuroprotective effect of PACAP is mediated by inhibition of ROS production by microglial cells (Yang et al., 2006). Moreover, PACAP is also able to increase the number of tyrosine hydroxylase (TH) immunoreactive neurons, and enhance dopamine uptake. Since PACAP acts as a neuromodulator, regulating synaptic transmission, its neuroprotective actions in PD may occur by regulation of dopamine release. Really, PACAP

induces catecholamine release from adrenal chromaffin cells, sympathetic neurons, and neurosecretory cells by elevating intracellular Ca^{2+} concentrations (Przywara et al., 1996; Ghzili et al., 2008; Mustafa et al., 2010). Different PD animal models have been developed to study this disease *in vivo* such as 1methyl-4-phenyl-1,2,3,6-etrahydropyridine (MPTP)-intoxicated mice. MPTP is a commonly used neurotoxin to produce experimental models of PD. It is responsible of energy depletion and dopaminergic neuronal loss in the SN, by inhibition the mitochondrial respiratory chain (Gerlach and Riederer 1996; Kostrzewa and Segura-Aguilar 2002). MPTP-intoxicated mice treated with PACAP improve memory impairment in the test session of the spatial reference version of the water maze (Masuo et al., 1993). Neuroprotective effects of PACAP in MPTP-induced PD mouse models may occur in different ways, such as by modulation of K (ATP) subunits and D2 receptors in the striatum or by in volving cholinergic neurotransmission, to balance the dopamine-acetylcholine systems in the basal ganglia neuronal pathway (Wang et al., 2008). Furthermore, PACAP is able also to act on the MPTP-altered expression of proteins, such as the mTOR anti-apoptotic and RNA-dependent protein kinase (PKR) apoptotic pathways of translational control (TC) (Deguil et al., 2007; 2010). Moreover, induction of unilateral lesion of the dopaminergic cells by 6-hydroxydopamine (6-OHDA) is also widely used to produce PD rodent models (Deumens et al., 2002). Injection of PACAP into 6OHDA-induced lesions in the SN protects dopaminergic nigrostriatal neurons from apoptosis, effectively reducing the dopaminergic neurodegeration in the SN and ventral tegmental area and improving behavioral symptoms (Reglodi et al., 2004; 2004). In addition, the role of PACAP in memory formation and consolidation may be a very important additional beneficial effect not only in AD but also in memory impairment observed in PD (Borbely et al., 2013; Pirger et al., 2014). Finally, PACAP, for its demonstrated antidepressant activity (Reichenstein et al., 2008) may help the Parkinson's patients which often suffer from depression (Grover et al., 2015).

5.3.5 Multiple Sclerosis

MS (multiple sclerosis) is a chronic autoimmune neurodegenerative disease of the CNS, afflicting over two-and-a-half million people worldwide. MS is characterized by an extensive and complex immune response, demyelination, glial scarring (sclerosis), perivascular leucocyte infiltration, and oligodendrocyte and axonal loss (Peterson and Fujinami 2007). Although it is not clear the cause that initiate disease MS, studies support that it is an autoimmune disease, regulated by APCs (antigen-presenting cells) and auto-reactive Th1 and Th17 cells (Steinman 2001; Sospedra and Martin 2005; Korn et al., 2009). These cells produce of inflammatory cytokines and chemokines in the CNS parenchyma and recruit macrophages, mast cells, neutrophils, $CD8^+$ T-cells and B-cells, which subsequently attack components of the CNS, including the myelin sheath and axons (Siatskas and Bernard 2009). The axonal damage accumulated determines a permanent loss of neurological function (Lassmann et al., 2007).

Experimental Autoimmune Encephalomyelitis (EAE), a widely model for MS research, is induced by immunization with a myelin peptide fragment such as MOG_{35-55} (amino acids 35-55 of myelin oligodendrocyte glycoprotein), thus resulting in the generation of autoreactive T-cells, which subsequently invade the CNS to initiate EAE, eventually culminating in myelin destruction (Waschek 2013). Due its immunological nature, strategies to treat MS are focused on suppressing inflammation. VIP and PACAP have recently been shown pronounced anti-inflammatory actions when administered systemically in animal models of inflammatory disease, including EAE (Abad et al., 2001, 2003; Delgado and Ganea 2001; Kato et al., 2004; Gonzalez-Rey et al., 2006b). The immunosuppressive effects of VIP and PACAP have been attributed to their capacities to inhibit antigen-specific Th1-driven responses and to modulate macrophage and dendritic cell (DC) function (Delgado et al., 2004b). It has been demonstrated that these peptides may modulate the production and/or activities of Treg (regulatory T) and Th17 cells (Chorny et al., 2005; Juarranz et al., 2005; Fernandez-Martin et al., 2006). Moreover, patients with MS have reported alterations in components of the VIP/PACAP signalling system, like observed in other inflammatory diseases (Tornwall et al., 1994; Belai et al., 1997; Boyer et al., 2007; Juarranz et al., 2008). They present decreased levels of VIP in their cerebral spinal fluid (Andersen et al., 1984; Sharpless et al., 1984), aberrant regulation of VPAC2 receptors in lymphocytes when stimulated *in vitro*, and a distinct DNA footprinting pattern in the promoter region of the VPAC2 gene (Sun et al., 2006). PACAP administration is able to ameliorate both the clinical and pathological manifestations of EAE. Splenocyte cultures from these mice exhibited significantly reduced MOG-specific IFN-g production. Treatment for three days with either VIP or PACAP was found to ameliorate EAE in a relapsing-remitting model, associated with decreased spinal cord levels of several proinflammatory cytokines, chemokines and chemokine receptors, and increased levels of the anti-inflammatory cytokines IL-10, IL-1Ra and TGF-β. Furthermore, the EAE phenotype of PACAP- and VIP-deficient (KO) mice (C57BL/6) using the $MOG_{35–55}$ model was also characterized (Tan et al., 2009; Abad et al., 2010). Clinical disease in PACAP KO mice was much more severe than in WT mice, involving four limb paralyses. The increased sensitivity was accompanied by up-regulation of mRNA expression of Th1 and Th17 cytokines in the spinal cord, and down-regulation of Th2 cytokines. In addition, the abundance of $CD4^+CD25^+FoxP3^+$ Tregs in lymph nodes and levels of FoxP3 mRNA in the spinal cord were reduced (Korn et al., 2007). All these data show an important promising role of PACAP in MS and also in other immunological pathologies.

6. AIM OF RESEARCH

The high impermeability and selectivity of the BBB prevents the transport of many therapeutic molecules into the brain and thus make their administration for the treatment of neurological disorders ineffective (Bernacki et al., 2008; Alyautdin et al., 2014). The development of new strategies to deliver therapeutic agents across the BBB is fundamental for the treatment of several brain disorders (Dominguez et al., 2014; Xu et al., 2013). The rapid evolving field of nanomedicine, permitted the production of new classes of highly potent drugs and imaging agents. However, the physicochemical and/or biological features of drugs do not permit to use them in humans. The nanosized delivery systems can circumvent these problems by improving the therapeutic index (Bertrand et al., 2012; Dinda et al., 2014). Between nanocarriers, liposomes have already had a major impact on many biomedical areas as drug delivery systems. Several liposomes have been approved for treatment of different pathologies while others are in clinical trials or on the market (Bozzuto and Molinari 2015). The most advantageous features of liposomes are their ability to incorporate and deliver large amounts of drugs and the possibility to decorate their surface with different ligands such as antibodies, peptides/proteins and carbohydrates (Sercombe et al., 2015). Recently, short cationic and/or amphipathic peptides, known as cell-penetrating peptides (CPPs), have been used to mediate the drug delivery (Drin et al., 2003; Farkhani et al., 2014; Wang et al., 2014; Capolovici et al., 2014). They are able to transport all types of macromolecules across the membrane bilayer *in vitro* and *in vivo* and other supramolecular aggregates (Heitz et al., 2009; Vivès et al., 1997; Angeles-Boza et al., 2010). Although their mechanism of uptake is not still known, the endocytosis seems the main pathway involved (Jones 2008). The endocytosis may represent a problem because of cargo endosome entrapment, which decreases the intracellular bioavailability. Membranotropic CPPs represent particularly attractive tools for drug delivery since their direct translocation across membranes which make the cargo transported immediately available in the cytosol, avoiding the endosomal entrapment and lysosome degradation (Galdiero et al., 2013). The peptide gH625, identified as a membrane-perturbing domain in glycoprotein H (gH) of *Herpes Simplex virus* 1, is able to traverse the membrane bilayer and deliver several variety of small molecules as well as proteins across cell membranes *in vitro* (Falanga et al., 2011; Smaldone et al., 2013; Tarallo et al., 2011; Guarnieri et al., 2013; Carberry et al., 2012). Although several studies have demonstrated that gH625 is an efficient carrier for bioactive cargoes *in vitro* (Falanga et al., 2013), these results do not guarantee that gH625 can be developed into a useful pharmaceutical delivery platform. On the basis of this interesting background, the aim of this Ph.D. project was to evaluate if gH625 can be used to develop efficient nanocarriers to deliver therapeutic agents to the brain.The project was divided in two parts. In the first part, *in vitro* and *in vivo* experiments were performed to investigate the capacity of gH625 to enter and accumulate in neuron and astrocyte cell lines, and its ability to cross the blood-brain barrier in rats. In the second part of project was evaluated the efficacy of liposomes functionalized with gH625 to transport a therapeutic

molecule across an *in vitro* model of rat BBB and *in vivo* in mice. For the experiments of *in vitro* BBB model the liposomes were loaded with PACAP (pituitary adenylate cyclase-activating polypeptide), a neurotrophic and neuroprotective peptide proposed for treatment of CNS injuries, stroke, and neurodegenerative diseases (Lee and Seo 2014), which shows a rapid degradation in the blood and limited accumulation in the brain (Meredith et al., 2015). Experiments of transport, uptake and toxicity were performed on *in vitro* model of rat BBB using different concentration of PACAP encapsulated in the liposomes. For the *in vivo* experiments we used liposomes loaded with VH-N439, a hypothermic neuropeptide currently developed by Vect-Horus as first-in-class neuroprotective agent acting in CNS (Jacquot G, Lécorché P, Khrestchatisky M. Activated neurotensin molecules and the uses thereof. Patent Application WO 2015/107182). This compound has been extensively studied in rodents where it showed dose-dependent hypothermic effect. VH-N439 was used here as a read-out of CNS delivery, as assessed by measuring the body temperature in mice, in order to compare the efficiency of BBB-crossing of gH625-liposomes compared to non-functionalized liposomes after intravenous injection. The results of this study could be interesting not only for the treatment of neurological diseases but also to provide an effective imaging and/or diagnostic tool.

7. MATERIALS AND METHODS

All experiments are realized in collaboration with Prof. Stefania Galdiero, Department of Pharmacy, University of Naples "Federico II". The experiments with liposomes are realized in the UMR7259-NICN laboratory supported by the CNRS and Aix Marseille University (AMU) and in collaboration with VECT-HORUS, a French biotechnology company that designs and develops vector molecules and vectorized drug candidates, under the supervision of Michel Khrestchatisky, director of the UMR7259-NICN laboratory.

7.1 Materials

7.1.1 gH625cys, PACAP-Rho

Fmoc-protected amino acid derivatives, coupling reagents, and Rink amide *p*-methylbenzhydrylamine (MBHA) resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Reagents (piperidine and pyridine) for solid-phase peptide synthesis were purchased from Fluka (Milan, Italy). Trifluoroacetic acid and acetic anhydride were from Applied Biosystems (Foster City, CA). H2O, N,N dimethylformamide, and CH3CN were supplied by Labscan Ltd. (Dublin, Ireland). 5(6)-Carboxytetramethylrhodamine N-succinimidyl ester (Rhodammina) and 4-Chloro-7-nitrobenzofurazan (NBD) were purchased from Sigma-Aldrich. All peptides were provided by Prof. Stefania Galdiero, Department of Pharmacy, University of Naples "Federico II", supported by Progetto FARO (2012/0043756).

7.2 Peptide synthesis

Peptides were synthesized using a standard solid-phase Fmoc (9-fluorenylmethoxycarbonyl) method as previously reported (Galdiero et al., 2005). All purified peptides were obtained with good yields (30–40%). The labeling with NBD-Cl (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) for gH625 and with Rhodamine (5(6)-Carboxytetramethylrhodamine N-succinimidyl ester) for PACAP27 were performed. Labeling was performed on resin-bound peptides as reported by Rapaport and Shai 2010. Peptides were fully deprotected and cleaved from the resin with trifluoroacetic acid. The crude peptides were precipitated with icecold ethyl ether, filtered, dissolved in water, lyophilized, and purified by preparative reverse-phase HPLC. The samples were eluted with a solvent mixture of H_2O and 0.1% trifluoroacetic acid (solvent A) and CH_3CN and 0.1% trifluoroacetic acid (solvent B). A linear gradient of 20–80% solvent B over 20 min at a flow rate of 20 ml/min was employed. The collected fractions were lyophilized to dryness and analyzed by mass spectrometry. For the synthesis of DSPE-PEG2000-gH625, DSPE-PEG2000-Mal (1 eq) was reacted with gH625Cys (1 eq) in DMF containing triethylamine (5 eq) for about 24 h. After the reaction completed, the solvent was evaporated by rotary evaporation and the

residues were redissolved in water, lyophilized and analyzed by RP-HPLC. The existence of the yielded products was confirmed by MALDI-TOF mass spectrometry (MALDI-TOF MS). The product was stored at -20◦C until used. The sequences of all synthesized peptides are reported in **Table 2**.

Peptide	Sequence	Molecular weight
gH625Cys	Ac-HGLASTLTRWAHYNALIRAF-Cys-$CONH_2$	2442.77
NBD-gH625Cys	NBD-HGLASTLTRWAHYNALIRAF-Cys-$CONH_2$	2564.77
DSPE-PEG2000-gH625	Ac-HGLASTLTRWAHYNALIRAF-Cys-PEG2000DSPE	5383.77
Pacap27	HSDGIFTDSYSRYRKQMAVKKYLAAVL-$CONH_2$	3147.63
RHO-Pacap27	RHO-HSDGIFTDSYSRYRKQMAVKKYLAAVL-$CONH_2$	3561.16

Table 2: **Sequence and molecular weight of peptides used.**

7.3 Liposomes preparation

Liposomes used for *in vitro* experiments are large unilamellar vesicles (LUV) consisting of DPPC/Chol (70/30) and DOPG /Chol (70:30) were prepared as previously reported (Galdiero et al., 2005). Lipid concentrations of liposome suspensions were determined by phosphate analysis (Fiske and Subbarow 1925). Briefly, various amounts of lipids and when necessary DSPE-PEG2000-gH625, PACAP-Rho and VH-N439 were dissolved in chloroform solution. The solvent was then removed with a nitrogen gas stream and lyophilized overnight. The lipid film was suspended in buffer by vortexing to produce LUVs. The lipid suspension was freeze-thawed eight times and then extruded 10 times through polycarbonate membranes with 0.1μm diameter pores (Northern Lipids). PACAP-Rho and VH-N439 encapsulated in liposomes were quantified by ultracentrifugation followed by HPLC analysis. All the preparations were provided by Prof. Stefania Galdiero, Department of Pharmacy, University of Naples "Federico II".

7.3.1 Particle size and zeta potential analysis

The hydrodynamic diameters (DH) and polydispersity index (PDI) of all liposomes were measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malven, UK). The analysis were performed with He–Ne laser 4 mW operating at 633 nm at scattering angle fixed at 173° and at 25°C. The results were determined three times for each sample and each

measurement was performed at least in triplicate. All liposome solutions present a monomodal distribution with a polydispersity index (PDI) < 0.2 indicating a narrow and homogenous size distribution **(Table 3).**

Liposomes	Averange Size (nm)	PDI
Liposomes loaded with PACAP	127.10±(10.65)	0.16±(0.14)
Liposomes loaded with PACAP and gH625 on the surface	173.60±(5.33)	0.23±(0.01)
Liposomes with VH-N439	114.40±(2.10)	0.07±(0.01)
Liposomes with VH-N439 and gH625 on the surface	106.3±(1.01)	0.14±(0.02)
Liposomes with gH625 on the surface	105.8±(0.66)	0.11±(0.01)

Table 3: Liposomes size and zeta potential analysis

7.2 Evaluation of gH625 peptide *in vitro*

7.2.1 Cell culture

The human neuroblastoma cell line (SH-SY5Y) was obtained from the American Type Culture Collection (Rockville, MD, USA). The human glioblastoma-astrocytoma cell line (U-87 MG) was kindly provided by Pasquale Barba (Institute of Genetics and Biophysics, Naples, Italy). Both cell lines (500000 cells) were seeded in 50 mL tissue culture flasks (25 cm^2 surface), and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) at 37°C under a humidified atmosphere of 5% CO_2 in air. The medium was changed twice a week, and cells were subcultivated when confluent.

7.2.2 MTT assay

The MTT assay was performed to evaluate the viability and metabolic state of the cells. MTT is taken up by cells and transformed into formazan by mitochondrial succinate dehydrogenase. Accumulation of formazan directly reflects the activity of the mitochondria, an indirect measurement of cell viability. In detail, in order to evaluate the effect of gH625 on the viability

of neurons and astrocytes, SH-SY5Y (at a density of 8000 cells/well) and U-87 MG (at a density of 5,000 cells/well) were seeded into 96 well-plates and incubated with increasing concentrations of peptide (0.5, 1, 5, 10, and 15 μM) for 20 hours. As negative controls, we used the phosphate-buffered saline. In particular, the same volumes (corresponding to the added peptide solutions) of the phosphate-buffered saline were added to the control wells; no significant differences were found. As a positive control, we added 400 or 800 μM of H_2O_2, which is known to induce cell death in these cell lines. At the end of each incubation, the medium was removed and the cells were incubated with MTT solution (0.5 mg/mL) for 3 hours at 37°C. The medium with MTT solution was then gently removed, and the formazan crystals were dissolved in isopropanol with, 0.1 M HCl. The optical density was detected with a microplate reader at 590 nm.

7.2.3 Fluorescence analysis

The SH-SY5Y and U-87 MG cell lines were then used to assess uptake of gH625. Cells were seeded at a density of 60,000/well on four-well chamber slides (x-well; Sarstedt AG & Co, Nümbrecht, Germany). Each well was treated with gH625 1 or 5 μM for 2 hours. The cells were then fixed in ice-cold methanol for 10 minutes and mounted with a coverslip in glycerol/phosphate-buffered saline 1:1. The cell nuclei were counterstained with DAPI (4′-6 diamidino-2-phenylindole) dilactate (Life Technologies). The slides were then analyzed using an epifluorescent microscope (Axioskop; Zeiss, Oberkochen, Germany), mounting the 40× objective with a numerical aperture of 1.3. Images were acquired using an AxioCam MR c5 (Zeiss) with differential interference contrast, a fluorescein isothiocyanate channel (λ_{ex} 488 nm; λ_{em} 515 nm) and a DAPI channel (λ_{ex} 350 nm; λ_{em} 461 nm).

7.2.4 Spectrofluorimetric analysis

To evaluate the percentage of internalized peptide, gH625 was administered at 1 or 5 μM for 2 hours to SH-SY5Y and U-87 MG cells previously seeded (5×10^4 cell/well) for 20 hours in a white-bottomed 96-multiwell plate without serum, suitable for spectrofluorimetric analysis. After incubation, the cells were rinsed twice with phosphate-buffered saline for 5 minutes and then lysed with 200 μL/well of lysis buffer in phosphate-buffered saline (Tris-HCl 10 mM, ethylenediaminetetraacetic acid 5 mM, NaCl 200 mM, 0,2% sodium dodecyl sulfate, proteinase K 100 μg/mL) in order to release the internalized gH625. The cell lysate was filtered through a 0.45 μm nylon membrane filter and transferred to new wells. The plates were analyzed with an Infinite 200 M spectrofluorimeter (Tecan, Männedorf, Switzerland) equipped with a fluorescence filter (λ_{ex} 485 nm; λ_{em} 560 nm). The standard curve (R^2=0.965) was obtained with

different concentrations of gH625, ie, 0.625 μM, 1.25 μM, 2.5 μM, and 5 μM in order to obtain the accurate concentration of internalized peptide. Spectrofluorimetric assays were performed in biological and technical triplicates.

7.3 Evaluation of gH625 peptide *in vivo*

7.3.1 Animals

Male Wistar rats (250-300 g) living in a temperature-controlled room at 24°C were kept, one per cage, under an artificial lighting regime of 12 hours light and 12 hours darkness. A commercial mash was available ad libitum and the animals had free access to water. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at the University of Napoli Federico II (Permit 2012/000180). All efforts were made to minimize suffering. The rats were divided in two groups, each consisting of three rats: one group received a single intravenous administration of vehicle and the other group received a single intravenous administration of gH625-NBD at a dose of 40 μg/100 g body weight or vehicle. The rats were anesthetized by intraperitoneal administration of Zoletil® 40 mg/100 g and then euthanized by decapitation 3.5 hours after administration of gH625-NBD. The liver and brain were rapidly dissected sagittally and divided in two symmetrical parts. One part was immediately processed for preparation of homogenate and the other was used for histological analysis.

7.3.2 Fluorescence histological analysis *ex vivo*

As soon as the brains and livers were dissected, they were fixed in Bouin's fluid (71% picric acid, 5% acetic acid, 24% formaldehyde) for 24 hours and embedded in paraffin wax (Carlo Erba, Milan, Italy). Sagittal 10 μm brain sections and 7 μm sections of liver were treated with 70% ethanol supplemented with 0.1% Sudan Black B for 20 minutes to minimize autofluorescence of tissues. The slides were then washed three times for 5 minutes with Tris-buffered saline containing 0.02% Tween 20 to remove the excess Sudan Black B (Baschong et al., 2001; Viegas et al., 2007). Sections were then stained with hemallum to evaluate tissue morphology. After dehydration, the sections were mounted with DPX mountant (Sigma-Aldrich). Images were acquired using the AxioCam MR c5 in bright field with a 488 nm ex/515 nm em filter. Images were then analyzed using ImageJ 1.48 software (Vonesch and Unser 2008).

7.3.3 Immunofluorescence analysis

Brain sections were dewaxed and hydrated. Antigen unmasking was performed with citrate buffer (pH 6.0) twice for 10 minutes in a microwave at 96°C (for anti-glial fibrillary acidic protein [GFAP] antibody, see below). Immunolocalizations were carried out with primary antibodies (negative technical controls were obtained by omitting primary antibodies) against specific endothelial protein found in areas with BBB (mouse monoclonal [SMI-71] anti-BBB), anti-GFAP (Abcam, Cambridge, MA, USA), or anti-tubulin III to determine if the gH625-NBD signal colocalized with brain endothelial cells, astrocytes, or neurons, respectively. To visualize antibodies, two secondary antibodies were used, i.e. Dylight 594 (Abcam) to detect anti-BBB and AlexaFluor 594 (Life Technologies) to detect anti-GFAP and anti-tubulin III, respectively. Images were acquired using the AxioCam MRc5 with λ_{ex} 488 nm/λ_{em} 515 nm or λ_{ex} 594 nm/λ_{em} 618 nm filters. Five images for each experimental class were then analyzed with ImageJ 1.48 software or Zen 2012; the Deconvolutionlab plugin was used to deconvolve image channels using the Tikhonov-Miller algorithm (Vonesch and Unser 2008); the Colocalization Colormap plugin was then used to evaluate the degree of correlation between pairs of pixels in the red and green channels, resulting in distribution of the values for the normalized mean deviation product (nMDP) and the index of correlation as the fraction of positively correlated pixels in the image (Jaskolski F et al., 2005). To evaluate the green fluorescence inside and outside the BBB vessels, the mean gray values of ten sections of controls (vehicle or gH625 without NBD; n=3) and treated animals (n=3) were measured within and outside the region of interest (ROI) of the BBB vessels. These values were then divided for the correspondent ROI area (μm^2) and the ratio between the outside and inside values was indicated. The two-tailed unpaired *t*-test was used, and the values were considered statistically significant at $P<0.01$.

7.4 Evaluation of gH625-liposome on rat *in vitro* BBB model

7.4.1 Primary rat brain endothelial cells co-cultured with astrocytes

Primary cultures of astrocytes and rat brain capillary endothelial cells (RBCEC) were performed as reported in Molino et al., 2014. Primary cultures of astrocytes were prepared from newborn rat cerebral cortex. The brain tissue was forced gently through a 70 μm nylon sieve, after removal of the meninges. Dissociated glial cells were seeded into cell culture flasks (75 cm^2 surface) and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 units/mL antibiotic penicillin, and 100 μg/mL streptomycin. After three weeks, astrocytes were trypsinized, frozen in liquid nitrogen and defrosted only 5 days before the establishment of the RBCEC co-culture at density of 80×10^3 cells per well of a twelve-well plate. RBCEC were obtained after isolation of brain microvessels from cortex of 3 five week old Wistar rats. The cells were seeded on type IV collagen and fibronectin (both 0.5 $\mu g/cm^2$;

BD) on Millipore filters (polyethylene twelve-well, pore size 1.0 μm) at density of 1.6×10^5 cells/cm^2 and maintained in DMEM/Ham's F12 supplemented with 20% bovine platelet-poor plasma-derived named endothelial cell media (ECM) (CliniSciences, Nanterre, France), 2 ng/mL bFGF, 100 μg/mL heparin (Life Technologies), 50 μg/mL gentamicin (Life Technologies) and 2.5 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; Life Technologies). After two days, the filters were transferred into the wells containing the astrocytes and the culture medium was replaced with ECM supplemented with hydrocortisone at 500 nM for differentiation and expression of junction-related proteins. Under these conditions, the *in vitro* models were used within 3 days. Liposomes alone or functionalized with gH625 and encapsulating PACAP-Rhodamine (gH625_LipoPACAP-Rho, LipoPACAP-Rho) were tested on rat *in vitro* BBB model to evaluate their penetration across cell monolayers, binding/ uptake on RBCEC and toxicity.

7.4.2 Penetration of liposomes across cell monolayers

To evaluate liposomes penetration across RBCEC cell monolayer, gH625_LipoPACAP-Rho and LipoPACAP-Rho were incubated for 60 minutes at 37°C at 5μM or 1μM PACAP38-Rho. After incubation, the medium was collected from the upper and lower compartments from each group and cell monolayers were rinsed twice with phosphate-buffered saline and then lysed with 500 μL/insert PBS 0.1% Triton X100 in order to release the internalized peptide. The fluorescence of medium and cell lysates were quantified by SpectraMax® M5e (Molecular Devices, Sunnyvale, CA, USA) equipped with a fluorescence filter (λ_{ex} 544 nm; λ_{em} 584 nm). Moreover, to evaluate the kinetic of passage across the RBCEC cell monolayer, liposomes were incubated for three different times (30, 60 and 120 minutes) at 37°C at 5μM PACAP38-Rho. The values of fluorescence were normalized using the equation of the standard curve for each class of liposomes. The standard curve were obtained with different concentrations of PACAP-Rho, i.e., 0.156 μM, 0.312 μM, 0.625 μM, 1.25 μM, 2.5 μM, and 5 μM in order to obtain the accurate concentration of peptide. As negative controls, the DMEM/Ham's F12 without phenol red was used. Each experiment was repeated five times.

7.4.3 Liposomes/PACAP uptake/binding

To assess the uptake or binding of PACAP/liposomes in RBCEC cell monolayer, gH625_LipoPACAP-Rho and LipoPACAP-Rho were incubated for 60 minutes at 37°C at 5μM and 1μM PACAP38-Rho. After incubation, the cells were fixed for 15 minutes with a 4% (w/v) paraformaldehyde solution followed by cutting of the insert membranes and nuclei staining with Höechst 33258 (Invitrogen). The membranes were washed three times with phosphate-buffered

saline and then mounted on microscope slides. The analyses of slides were performed using confocal microscopy LSM 780 (Zeiss, Jena, Germany) equipped with a 63x oil-immersion objective, and ZEN Software 2012. Images were acquired using filters for rhodamine channel (λ_{ex} 544 nm; λ_{em} 584 nm) and Höechst channel (λ_{ex} 350 nm; λ_{em} 461 nm).

7.4.4 Lucifer yellow assay

Lucifer yellow assay was performed to evaluated the monolayer tightness during the experiments and absence of toxicity of liposomes. This assay allows to measure the transport of lucifer yellow (LY-CH, dilithium salt; Sigma-Aldrich), a small hydrophilic molecule (MW 457 Da) known to be retained by the BBB (Molino et al., 2014). The inserts containing the RBCEC monolayer were gently washed and transferred to clean twelve-well plates. Both the upper and lower chambers were washed with pre-warmed DMEM/F12 without phenol red. LY was co-incubated with the different classes of liposomes (gH625-LipoPACAP or LipoPACAP) at 5 µM of PACAP in the upper compartment of the culture system, in contact with cerebral endothelial cells, for 60 min at 37°C. After this time, the medium of the lower compartment was collected and fluorescence was quantified by fluorimetric analysis with the SpectraMax® M5e (Molecular Devices, Sunnyvale, CA, USA) equipped with a fluorescence filter (λ_{ex} 430/485 nm; λ_{em} 535 nm). The analysis of values was performed as reported in Molino et al, 2014.
The results are expressed in permeability coefficient (Pe) in 10^{-3} cm/min. The barrier is considered permeable or open when the Pe of LY is above $0.6x10^{-3}$ cm/min.

7.4.5 Immunocytochemistry

To evaluate tight junction morphology and as consequence the toxicity of liposomes, immunocytochemistry for zonula occludens (ZO1)-1 was performed. gH625-LipoPACAP and LipoPACAP, at 5 µM of PACAP38, were incubated with RBCEC for 60 minutes at 37°C. After incubation, the membranes of filters containing the endothelial cells were washed twice with phosphate-buffered saline and fixed for 15 minutes with a 4% (w/v) paraformaldehyde solution. Then the cells were permeabilized for 10 minutes with PBS 0.1 % Triton X100 followed by washing with PBS twice. Blocking was done using 3% BSA for 30 minutes followed by wash with PBS and incubation with ZO1 antibody (Rabbit anti-ZO1 Zymed 61-7300) diluted 1:200 in a solution of 1% BSA for 1 hour at room temperature. After two washes, cells were incubated with secondary antibodies Alexa Fluor 488 (1:800) and Höechst (1:1000) for 30 minutes at room temperature, followed by three washes with PBS. Finally, cells were mounted with ProLong® Gold Antifade Mountant (Life Technologies) and visualized under confocal microscopy (LSM 780; Carl Zeiss, Jena, Germany) equipped with a 63x oil-immersion

objective by using filters for Alexa 488 channel (λ_{ex} 544 nm; λ_{em} 584 nm) and Höechst channel (λ_{ex} 350 nm; λ_{em} 461 nm).

7.5 Evaluation of gH625-liposome *in vivo*

7.5.1 Body temperature in mice

Swiss CD1 mice (25-30 g) were kept in a temperature-controlled room at 24°C, with free access to food and water, with 12-hour light/dark cycle. The study was carried out in strict accordance with the recommendations of, and approval by, the Ethics Committee of the Aix Marseille University and authorisation of the French ministry of Research granted to the NICN laboratory. The mice were divided in six groups, each consisting of 4 mice. VH-N439 or different classes of liposomes loaded with VH-N439 were injected intravenously at different doses (expressed as molar equivalent of the full length neurotensin, or "Eq. NT") (**Table 4**). All groups were injected with an initial dose of 3 mg/kg Eq NT, as this dose of the free VH-N439 was previously shown to induce a clear hypothermic effect in mice. Then a dose of 6 mg/kg Eq NT was used in order to assess the dose-response relationship of liposomal formulations. Empty gH625-functionalized liposomes were used as negative control. All liposomes contained the same lipid concentration. After injection of either the free VH-N439 of the different liposomal formulations, the body temperature was measured at different times during 4 hours.

Liposomes	Eq. NT
VH-N439	6.27×10^{-8} mol (3 mg/kg)
Lipo(VH-N439)	6.27×10^{-8} mol (3 mg/kg)
gH625-Lipo(VH-N439)	6.27×10^{-8} mol (3 mg/kg)
Lipo(VH-N439)	1.254×10^{-7} mol (6 mg/kg)
gH625-Lipo(VH-N439)	1.254×10^{-7} mol (6 mg/kg)
gH625-Lipo	

Table 4: **Molar equivalent quantity of neurotensin in liposomes.**

Statistical analysis

All data are expressed as mean ± SEM. For the data on rat BBB *in vitro* model the statistical comparisons were carried out between the groups using two sample *t*-test assuming unequal variances. A significance value of $P<0.05$ was accepted and considered as relevant. For the experiments in mice, a two-way ANOVA with a Bonferroni post-test was used to compare the pharmacodynamic profile (treatment and time effect) of each formulation.

8. RESULTS

8.1 Evaluation of gH625 peptide *in vitro*

8.1.1 MTT assay

The toxicity of gH625 in neuroblastoma cells (SH-SY5Y) and astrocytoma cells (U-87 MG) was tested using the MTT assay [3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide]. Incubation of SH-SY5Y cells (**Figure 15A**) and U-87 MG cells (**Figure 15B**) to gH625 at a concentration of 0.5-15 μM for 20 hours had no significant effect on cell viability. These results suggest that the peptide is not toxic to brain cell populations at the concentrations utilized.

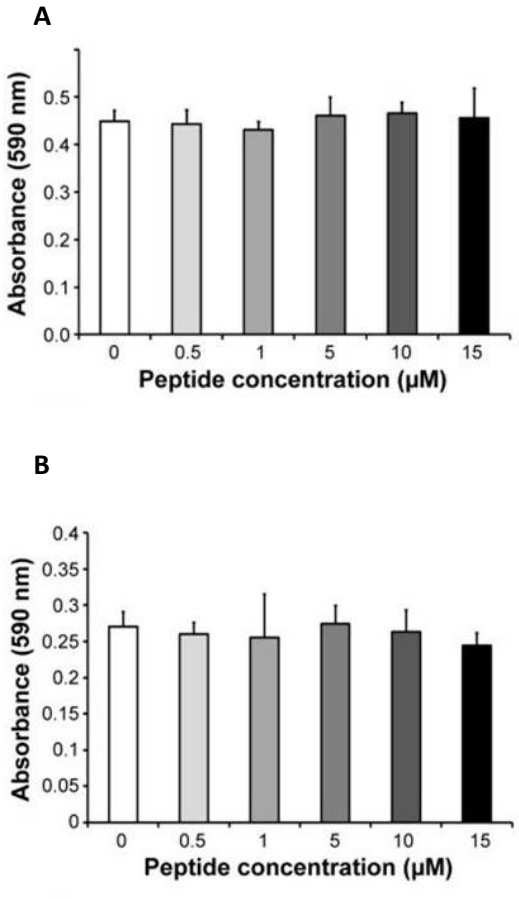

Figure 15: Effect of *in vitro* incubation of gH625 with SH-SY5Y cells and U-87 MG cells on cell viability. MTT assay for SH-SY5Y cells (**A**) and astrocytoma U-87 MG cells (**B**). Indicated as zero peptide in the figure is the negative control corresponding to the cells treated with vehicle (the same amount in μL of the 15 μM peptide concentration).

8.1.2 Fluorescence analysis

To confirm internalization of gH625, was examined its cellular uptake by fluorescence microscopy. SH-SY5Y and U-87 MG cells showed significant uptake of gH625 after 2 hours of incubation. The peptide was efficiently internalized in both cell lines (**Figure 16E and K**). NBD alone was almost unable to penetrate into the cells, as previously reported (Falanga A et al., 2011).

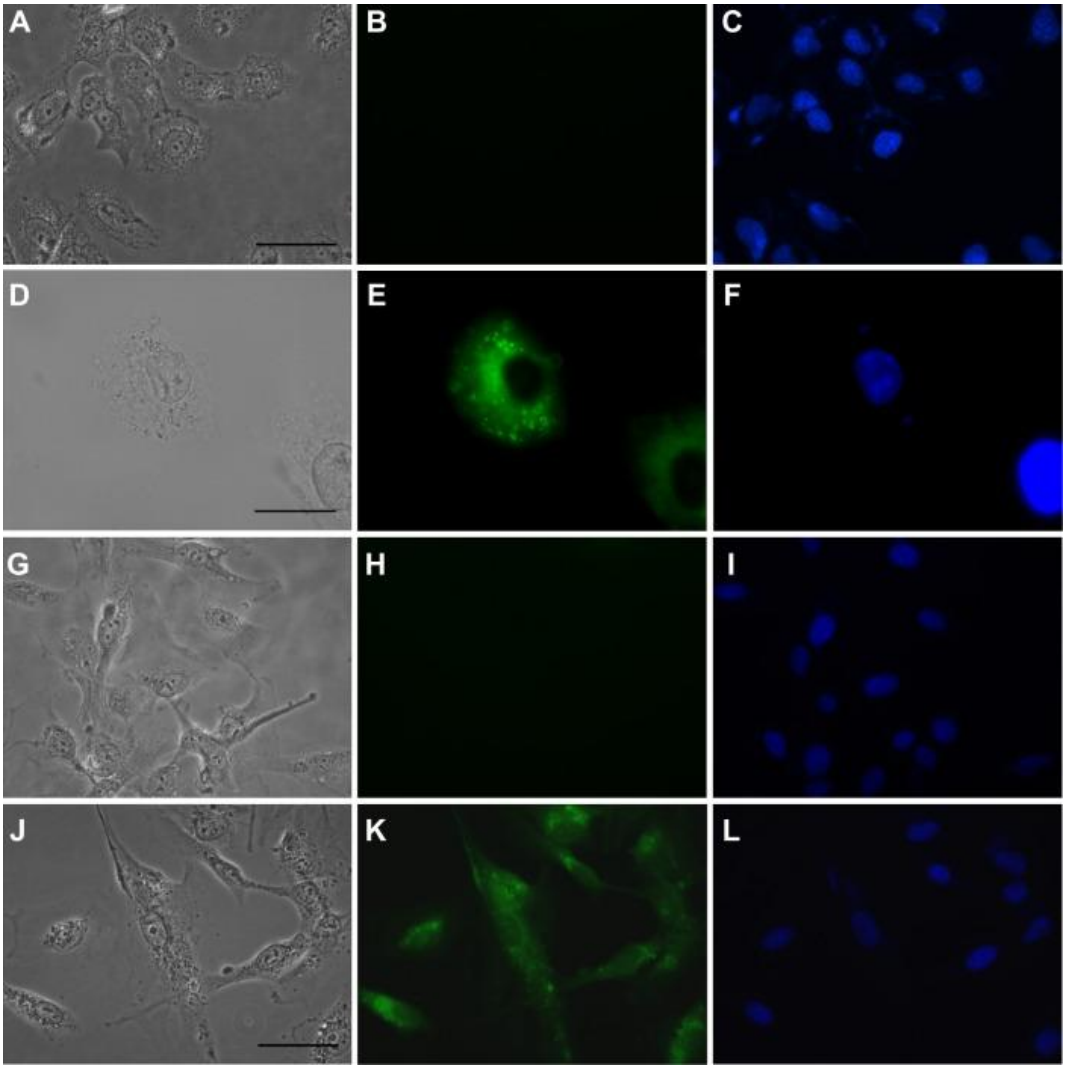

Figure 16: *In vitro* uptake of 5 μM gH625. The left column shows images acquired with differential interference contrast, the central column with the fluorescein isothiocyanate channel, and the right column with the 4′-6-diamidino-2-phenylindole channel. Each row represents the same field. (**A-C**) Negative control for SH-SY5Y cells. (**D-F**) SH-SY5Y cells treated with gH625. (**G-I**) Negative control for U-87 MG cells. (**J-L**) U-87 MG cells treated with gH625. Scale bars correspond to 50 μm, except for (**D-F**) where they correspond to 20 μm.

8.1.3 Spectrofluorimetric analysis

A spectrofluorimetric analysis was carried out to evaluate the uptake of gH625 in both cell lines in a quantitative manner. The data clearly confirm the results obtained by microscopy **(Figure 17)**. Indeed, SH-SY5Y (**Figure 17A**) and U-87 MG (**Figure 17B**) cells efficiently accumulated gH625. At low concentration (1 μM), approximately 30% of gH625 was internalized, while uptake was nearly complete (80%-90%) at a concentration of 5 μM.

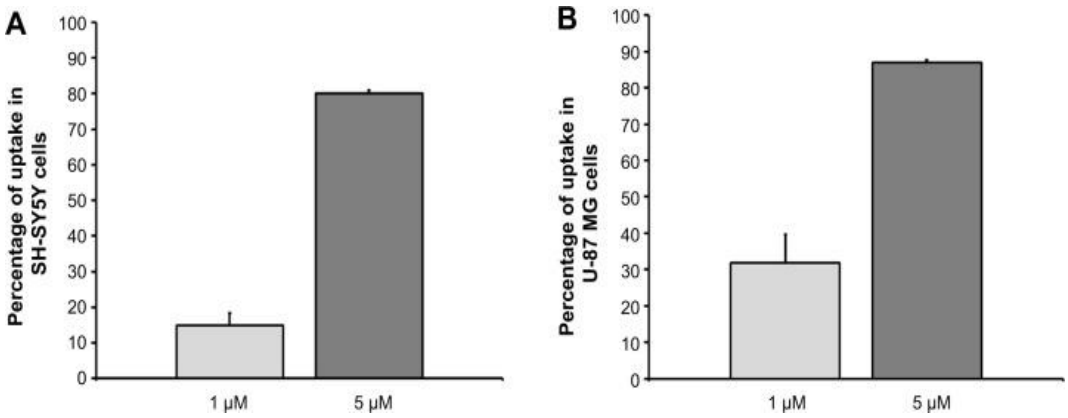

Figure 17: (**A**) Spectrofluorimetric analysis of SH-SY5Y cell uptake after exposure to gH625, 1 and 5 μM. (**B**) Spectrofluorimetric analysis of U-87 MG cell uptake after exposure to gH625, 1 and 5 μM.

8.2 Evaluation of gH625 peptide *in vivo*

8.2.1 Fluorescence histological analysis *ex vivo*

In order to perform the histological analysis, dissected brain and liver tissues were fixed and stained and images were acquired and analyzed. The fluorescence images clearly show that within 3.5 hours of its *in vivo* administration, gH625 was partly taken up by Kupffer cells in the liver, which are clearly distinguishable from hepatocytes because they possess the typical morphology of macrophages, and showed overfilled cytoplasm (**Figure 18C and D**). In addition, gH625 reaches the BBB and accumulates in the extensions of nerve cells (**Figure 18G and H**).

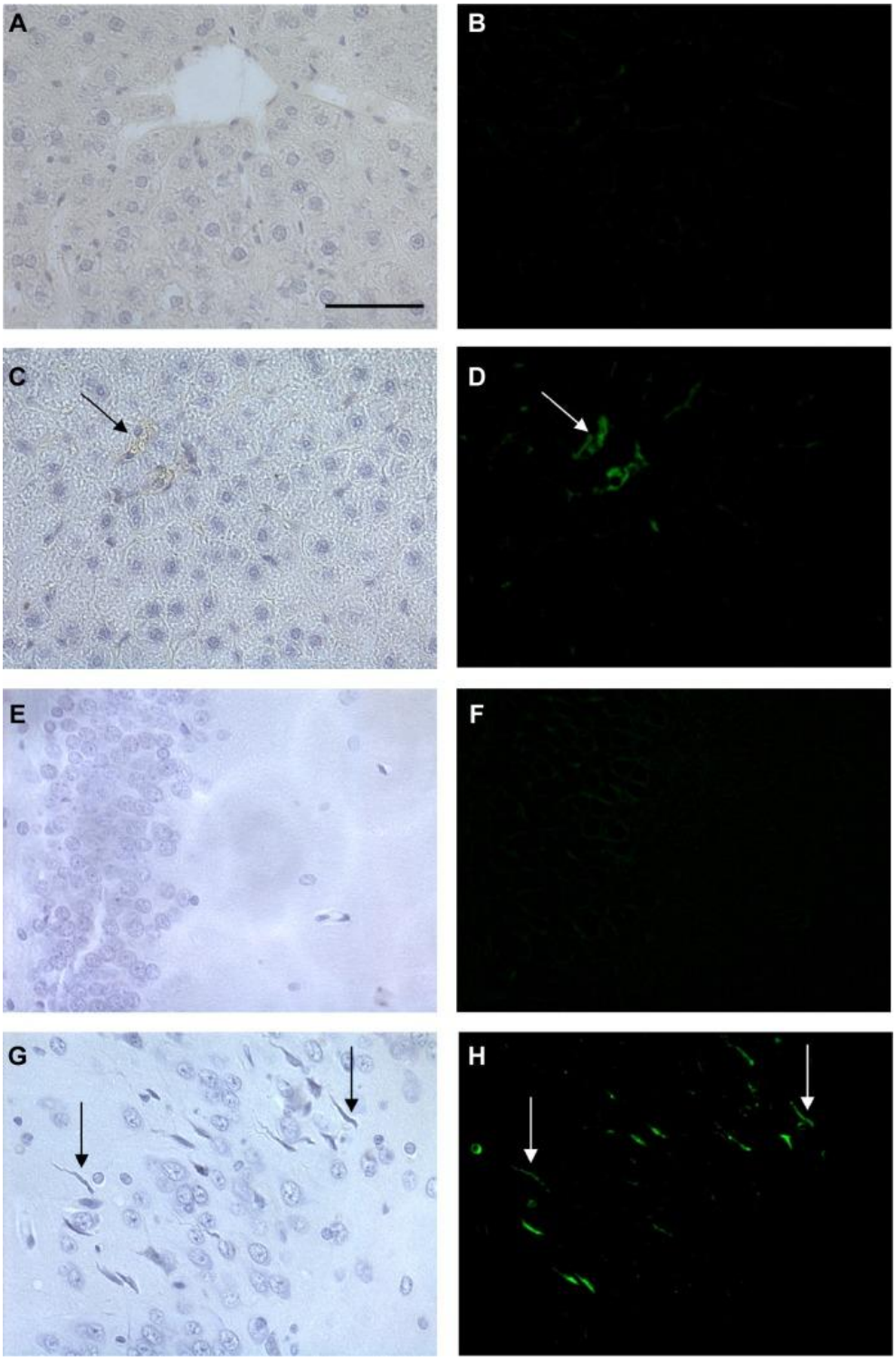

Figure 18: Representative histological images of the liver and brain in control rats and rats treated with 16 µM gH625. The left column shows bright-field images and the right column shows the fluorescein isothiocyanate channel. Each row represents the same field. The scale bar corresponds to 50 µm. (**A**, **B**) Liver section of negative control, (**C**, **D**) liver of treated animal showing Kupffer cell (arrows) overfilled with gH625, (**E**, **F**) a brain section from a negative control, and (**G**, **H**) brain of treated animals showing the presence of gH625 in nerve cell processes (arrows).

8.2.2 Immunofluorescence analysis

By evaluation of fluorescence levels, we demonstrated that gH625 passes significantly through the BBB compared to controls (**Figure 19**). Immunofluorescence demonstrated that gH625 accumulates in the blood vessels of the brain in treated rats. No signal was found in the negative control for the immunofluorescence technique (**Figure 20A-a4**). Low spurious fluorescent signals arose from scattered red blood cells in the control animals (**Figure 20B-b4**). There was little or no colocalization between gH625 and astrocytes surrounding the BBB, as shown by immunofluorescence and nMDP color images (**Figure 20C-c4**). There was no colocalization between gH625 and the BBB in the control animals (**Figure 20D-d4**). More colocalization was found between gH625 and the endothelial BBB on immunofluorescence and nMDP color images (**Figure 20E-e4**). It is of interest that some cells in the cerebral cortex, which morphologically and by immunofluorescence can be ascribed to neurons and their processes beyond the BBB were labeled for gH625, indicating the passage of gH625 through the BBB (**Figure 20F-f4, Figure 21**). The nMDP distribution values (**Figure 22A and C**) and the index of correlation (**Figure 22B and D**) suggest that gH625 labeling is correlated mainly with BBB than to astrocytes.

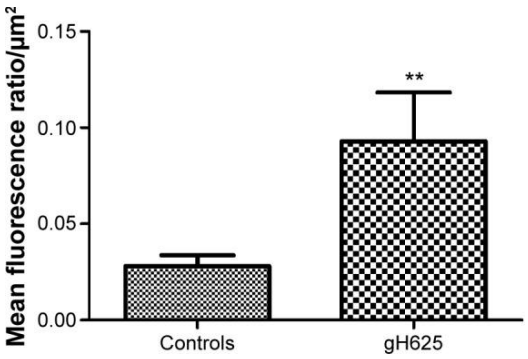

Figure 19: Green fluorescence evaluation of control and gH625-treated brain slices. The ratio of mean fluorescence outside and inside the blood vessels of the blood-brain barrier was calculated for at least ten slices (control animals n=3, treated animals n=3). ***P*<0.01.

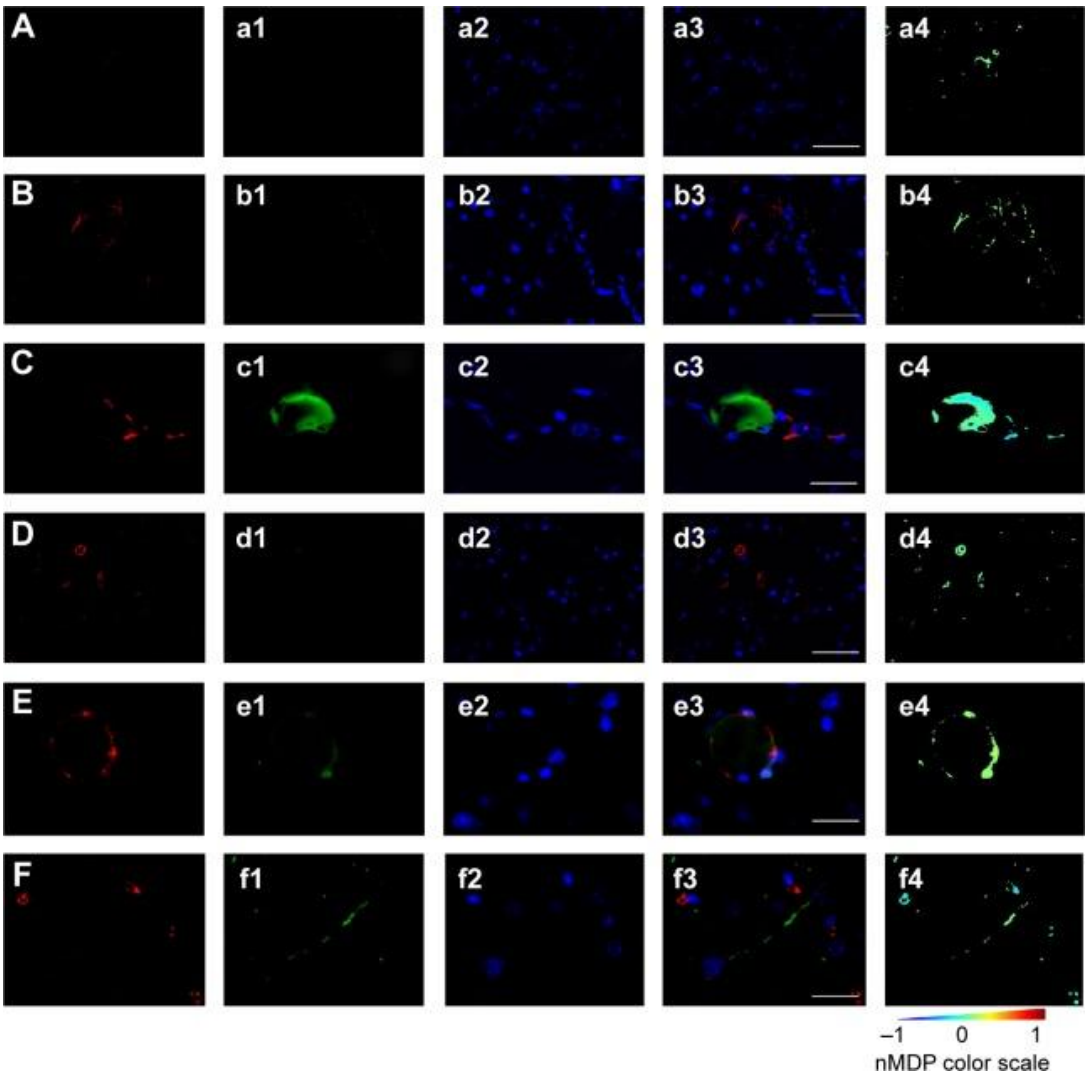

Figure 20: Representative images of brain immunofluorescence using anti-BBB and anti-GFAP in control and gH625-treated animals. From left to right, the columns show AlexaFluor594, fluorescein isothiocyanate, DAPI, and merged and colocalization map images, respectively. For the colocalization map (from -1 to 1) a color scale is provided: negative indices (cold colors) represent no colocalization, while indices above 0 (hot colors) represent colocalization. (**A-a4**) Negative control (omission of primary antibody) for anti-BBB immunofluorescence in control animal (vehicle or gH625 without NBD) showing low red background and no green signal (anti-GFAP negative control showed the same pattern and is not shown). Scale bar corresponds to 50 μm. (**B-b4**) Anti-GFAP immunofluorescence in a control animal. Scattered spots of low green signal were due to red blood cells. Scale bar 50 μm. (**C-c4**) Anti-GFAP immunofluorescence in a gH625-treated animal showing no overlapping of red and green signal. Scale bar corresponds to 20 μm. (**D-d4**) Anti-BBB immunofluorescence in a control animal. Scattered spots of low green signal can be attributed to red blood cells. Scale bar corresponds to 50 μm. (**E-e4**) Anti-BBB immunofluorescence in a gH625-treated animal showing overlapping of red and green signal. Scale bar corresponds to 20 μm. (**F-f4**) Anti-BBB immunofluorescence in a gH625-treated animal showing that the presence of gH625 beyond the BBB can be associated to nerve cell processes. Scale bar corresponds to 20 μm.

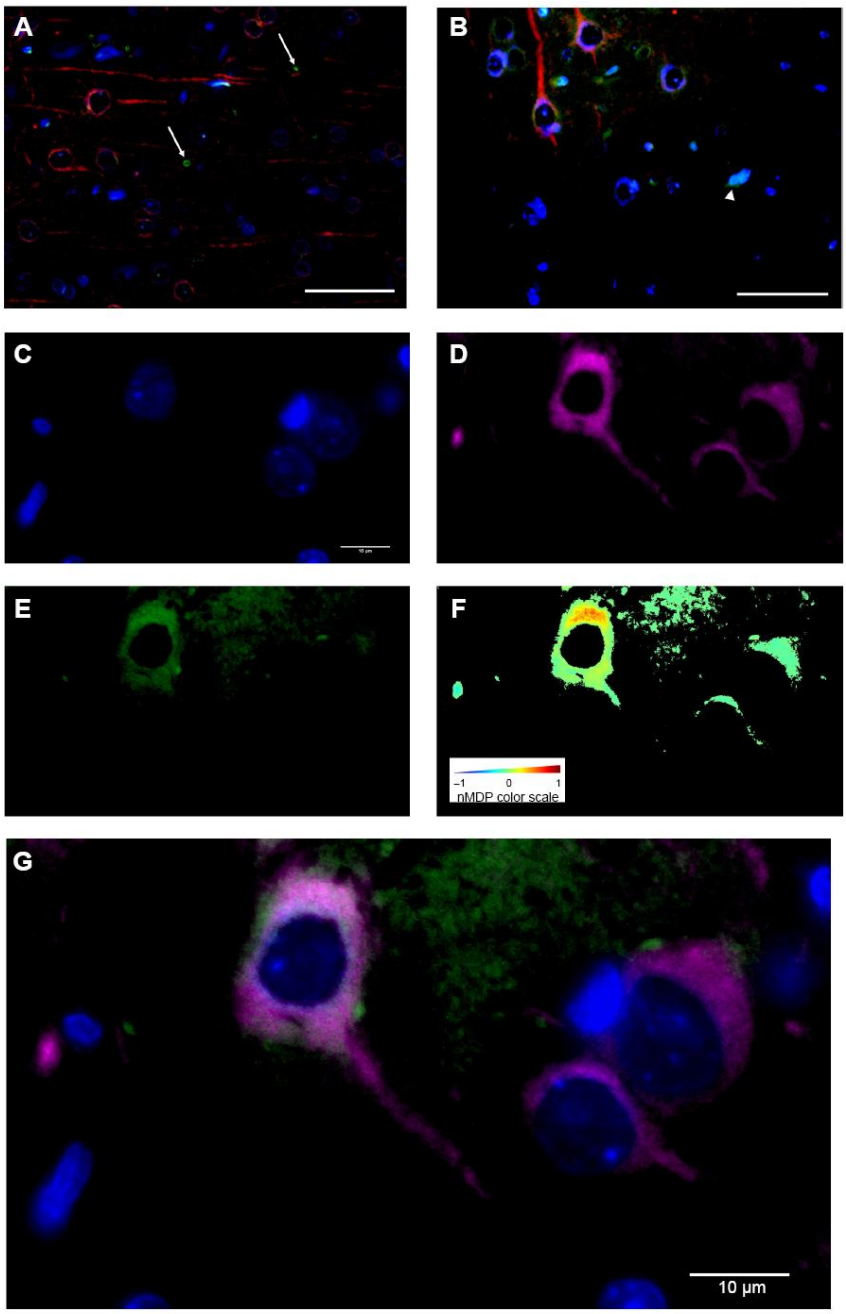

Figure 21: Immunofluorescence for anti-tubulin III on cerebral cortex sections from gH625-NBD-treated animals. (**A**) Neurons appear largely without labeling for gH625-NBD, while the peptide signal is restricted to narrow areas (arrows). (**B**) In some neurons, gH625-NBD colocalizes with anti-tubulin III near the perikaryon. Other cytotypes are also labeled for gH625-NBD (arrow head). (**C**) DAPI staining of neuron nuclei. (**D**) Neurons immunoreactive for anti-tubulin III (magenta: false color) in the perikaryon and axons. (**E**) The same field in the fluorescein isothiocyanate channel shows the same neuron in (**D**) which is strongly labeled for gH625-NBD. (**F**) nMDP distribution colocalization color map of overlapping (**D**) and (**E**); the image clearly shows that gH625-NBD colocalizes with anti-tubulin III in the perikaryon and the axon emergence, exclusively. (**G**) Merged image of the aforementioned three channels, ie, DAPI, AlexaFluor594, and fluorescein isothiocyanate. Scale bar: 50 µm for (**A**, **B**) and 10 µm for (**C**-**G**).

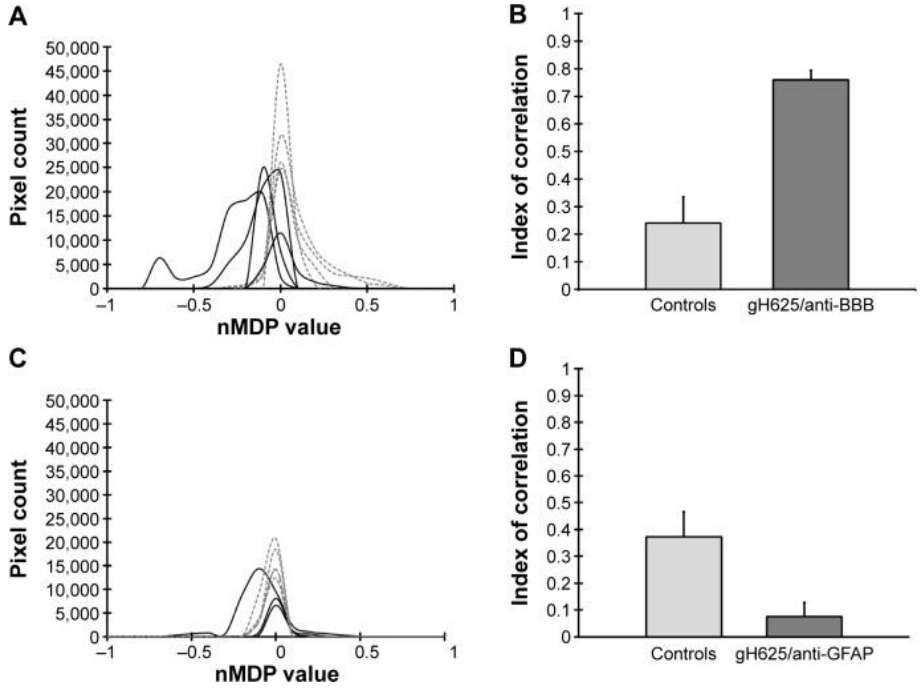

Figure 22: Pixel count and pixel correlation analysis on images from control and gH625 NBD treated experimental groups. Distribution of nMDP shows the degree of colocalization in the control, ie, vehicle or gH625 without NBD (solid lines) and gH625/anti-BBB (dashed lines) (**A**). The index of correlation histogram comparing control and gH625/anti-BBB images shows a high fraction of positively correlated pixels (**B**). Distribution of nMDP shows the degree of colocalization between the control (solid lines) and gH625/anti-GFAP (dashed lines) (**C**). The index of correlation comparison between control and gH625/anti-GFAP showing a low fraction of positively correlated pixels in the images (**D**).

8.3 Evaluation of gH625-liposome on rat *in vitro* BBB model

8.3.1 Penetration of liposomes across cell monolayers

For penetration studies, gH625_LipoPACAP-Rho and LipoPACAP-Rho were incubated at 5µM and 1µM PACAP-Rho with RBCEC. After different time of incubation the amount of dye was determined in the RBCEC lysate, upper and lower compartment and compared to the initial amount of dye incubated. Standard curve was performed for each class of liposomes and for PACAP-Rho free and used to obtain accurate concentration of PACAP-Rho in all compartments of BBB *in vitro* model. The results showed that the amount of PACAP-Rho in the upper compartment after 60 minutes decreased compared to the initial concentration of PACAP-Rho

(**Figure 23A**). The amount of PACAP-Rho in RBCEC was very low for both liposomes but significantly higher for Lipo than for gH625_Lipo (**Figure 23B**). At lower concentration (1 µM) the amount of PACAP-Rho in the lower compartment was very low (around 0.25 µM), without significantly difference between the two classes of liposomes. The liposomes penetration across BBB monolayer increased with the concentration but statistically significant only for the class gH625_Lipo. At higher concentration (5 µM), the amount of PACAP-Rho which crosses the monolayer was higher for gH625_Lipo than unfunctionalized liposomes (0.93 and 0.64 µM, respectively) (**Figure 23C**). The kinetic results, at 5 µM concentration, show that after each time of incubation the amount of PACAP-Rho in the upper compartment decreased compared to the initial concentration of PACAP-Rho (**Figure 24A**). PACAP-Rho in RBCEC was very low for both liposomes in each time, with significant difference only at 60 minutes (**Figure 24B**). The data of lower compartment indicated that the liposomes penetration was time-dependent. The PACAP-Rho amount which pass across the BBB monolayer was higher for gH625_Lipo than Lipo also in the first 30 minutes of incubation (0,72 and 0,60, respectively) (**Figure 24C**). After 120 minutes, the amount of PACAP-Rho detected in the lower compartment was higher compared to 30 minutes for both liposomes but without significant difference between gH625_Lipo and Lipo (**Figure 24C**). The data analysis on the lower compartment at 5 µM show that the amount of PACAP-Rho which crosses the BBB monolayer after 30 minutes of incubation is 19.4% higher when transported by gH625_Lipo than Lipo (**Figure 25A**) and 44.3% higher after 60 minutes (**Figure 25B**). Analysis of kinetic data revealed that PACAP-Rho transported across the BBB monolayer by gH625_Lipo increased of 29% from 30 minutes to 60 minutes and of 36,5% from 60 minutes to 120 minutes (**Figure 26**).

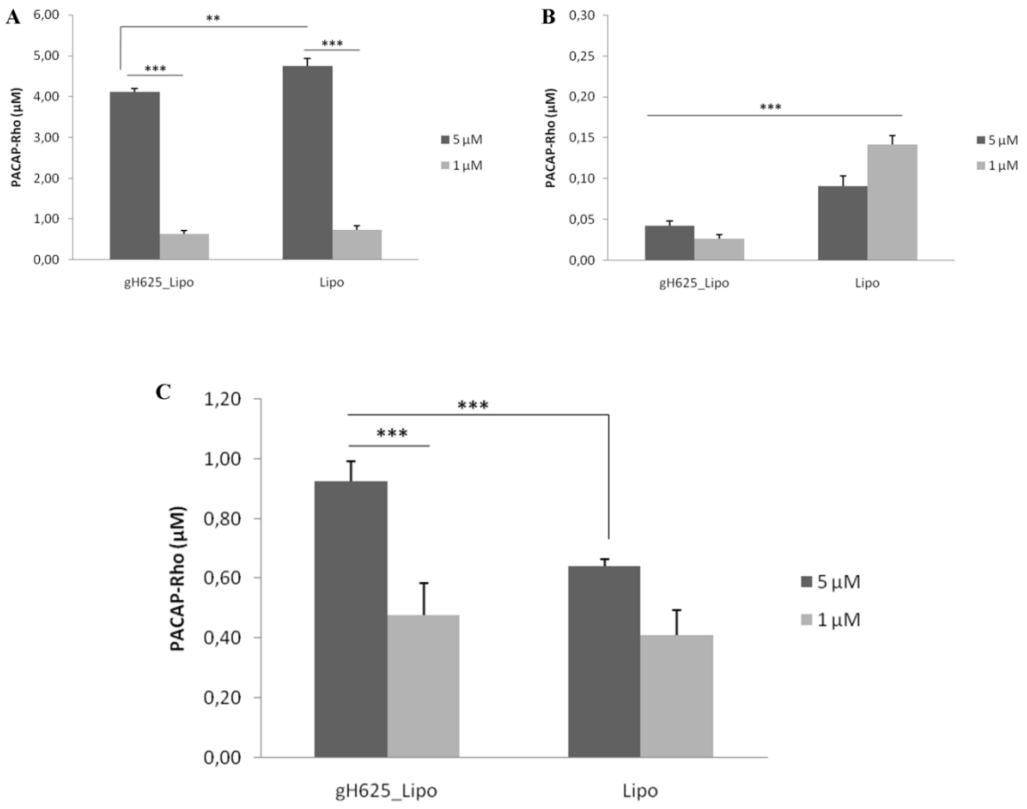

Figure 23: *In vitro* blood-brain barrier penetration at 1 and 5 µM PACAP-Rho after 60 minutes. (A) PACAP-Rho amount in the upper compartment; **(B)** PACAP-Rho in the RBCEC lysate; **(C)** PACAP-Rho in the lower compartment. Values represent means ± SEM of three independent experiments (**P < 0.005; ***P < 0.0005).

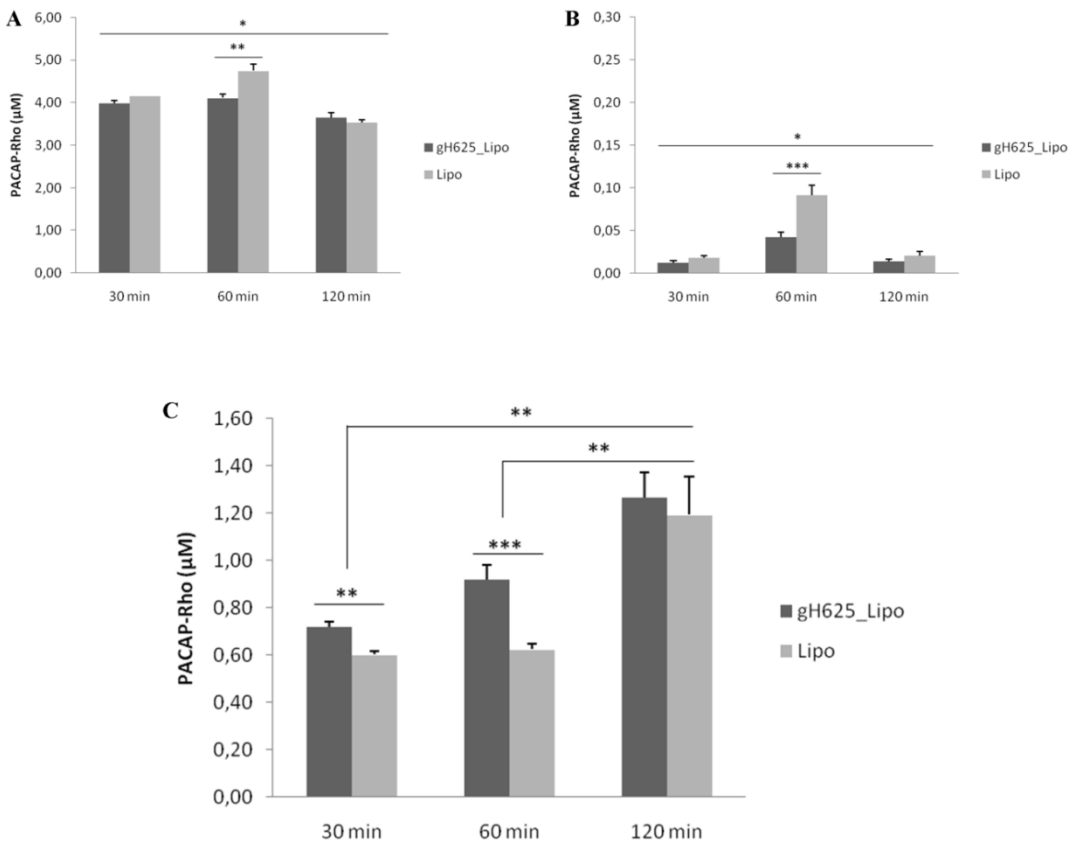

Figure 24: ***In vitro*** **blood-brain barrier penetration kinetic at 5 µM PACAP-Rho. (A)** Values of PACAP-Rho in the upper compartment; **(B)** PACAP-Rho in the RBCEC lysate; **(C)** PACAP-Rho in the lower compartment. Values represent means ± SEM of three independent experiments (*P < 0.05; **P < 0.005; ***P < 0.0005).

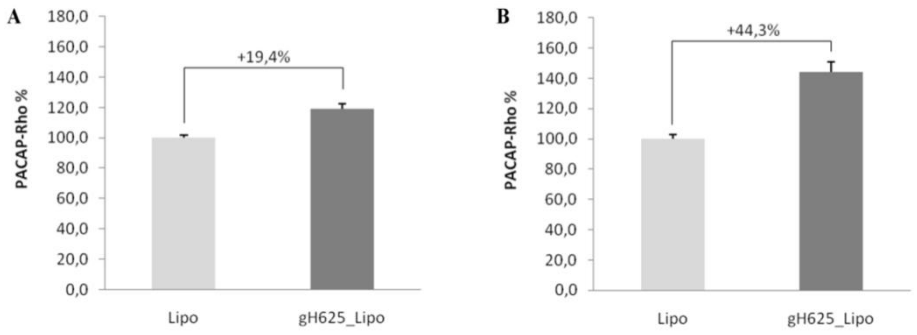

Figure 25: Percentage of PACAP-Rho in the lower compartment after 30 and 60 minutes. (A) Percentage of PACAP-Rho after 30 minutes charged liposome incubation at 5 µM; **(B)** after 60 minutes. The data are expressed as variation respect Lipo. Values represent means ± SEM of three independent experiments

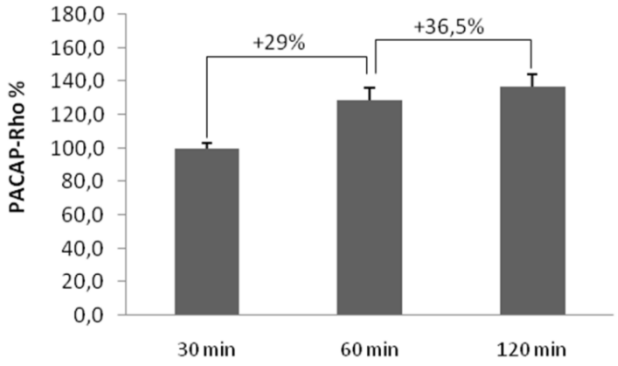

Figure 26: Percentage variation of PACAP-Rho transported by gH625_Lipo in different time. After incubation with gH625_Lipo, the amount of PACAP transported across the BBB monolayer increased of 29% from 30 minutes to 60 minutes and of 36,5% from 60 minutes to 120 minutes. Values represent means ± SEM of three independent experiments

8.3.2 Liposomes/PACAP uptake/binding

In the liposome-uptake/binding studies, the results showed a very low presence of PACAP-Rho in both experimental classes after 60 minutes of incubation. At 1 µM of PACAP-Rho there were no specific detectable signals in RBCEC. The representative fluorescence images at 5 µM of PACAP-Rho showed that the unfunctionalized liposomes have more binding/uptake to RBCEC compared to liposomes functionalized with gH625 (**Figure 27**).

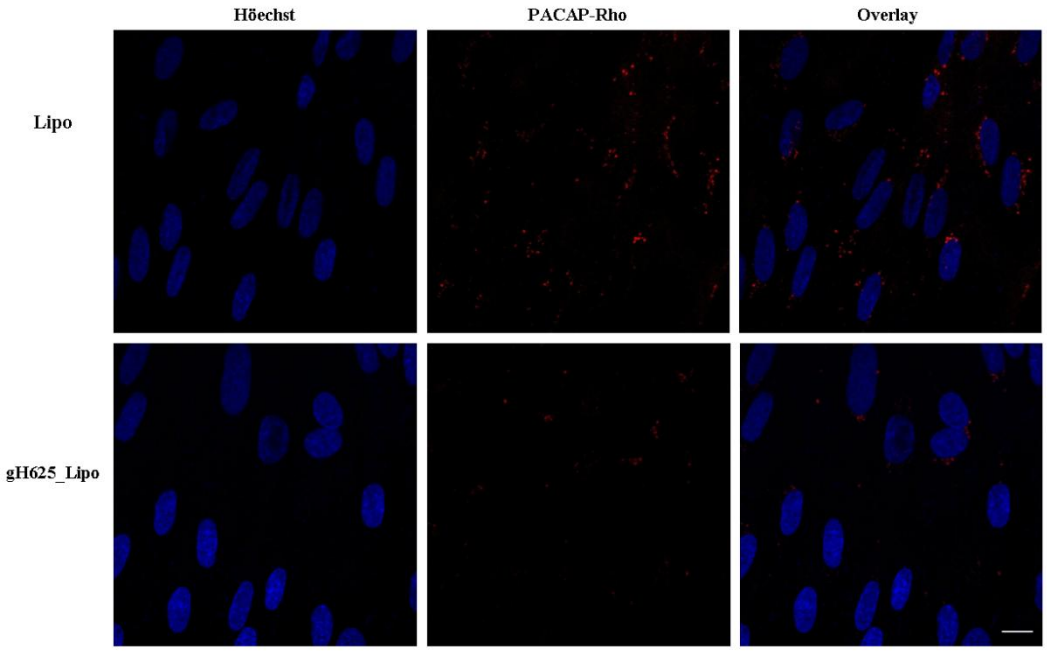

Figure 27: Liposomes/PACAP uptake/binding studies. The left column shows images acquired with the Höechst channel, the central column with the rhodamine channel, and the right column merged. Each row represents the same field. Scale bar corresponds to 10 µm.

8.3.3 Lucifer yellow assay

LY assay was used to evaluate the liposomes toxicity on endothelial cells. The results showed that after 60 minutes of incubation the values of permeability coefficient (Pe) of LY is in an average of $0.24 \pm 0.03 \times 10^{-3}$ cm/min for both liposomes. These values are similar to control obtained without liposomes ($0.25 \pm 0.03 \times 10^{-3}$ cm/min). The barrier is considered permeable or open when the Pe of LY is above 0.6×10^{-3} cm/min (Molino Y et al., 2014). Therefore, the LY assay on RBCEC showed that in neither of the cell inserts treated with liposomes alone and

liposomes functionalized with gH625 there was significant increase of endothelial permeability to LY (**Figure 28**).

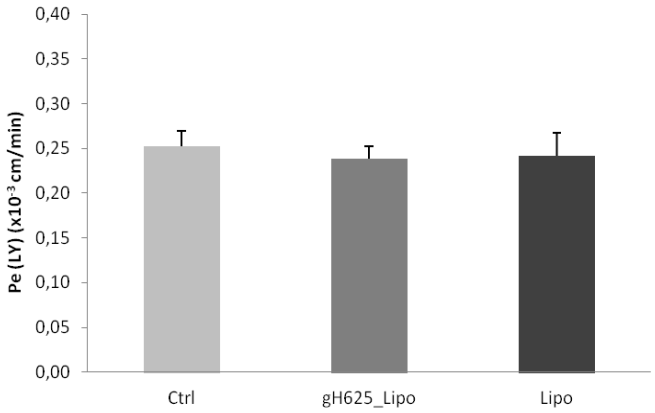

Figure 28: Lucifer yellow assay on RBCEC. The graph showing no significant impact of liposomes on endothelial cell permeability for LY compared to control (Ctrl: cells alone), indicating that liposomes caused no significant opening of the endothelial cell monolayer. Values represent means ± SEM of three independent experiments.

8.3.4 Immunocytochemistry for ZO1

As a complementary approach, the effect of liposomes on the expression and distribution of the tight junction marker ZO1 was assessed. The immunocytochemistry for RBCEC showed that after 60 minutes of incubation at 5µM of PACAP-Rho the liposomes (functionalized or not) caused no significant change in ZO1 distribution (**Figure 29**).

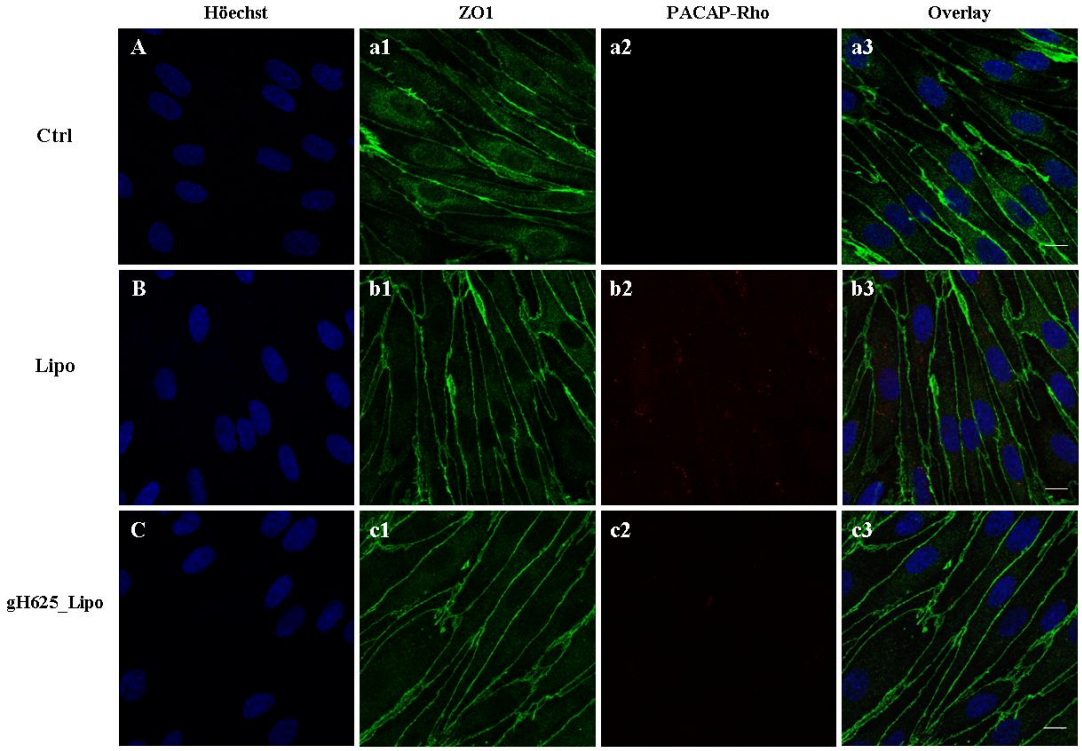

Figure 29: Immunocytochemistry for ZO1 on RBCEC after 60 minutes at 5 µM PACAP-Rho. From left to right, the columns show Höechst, fluorescein isothiocyanate, rhodamine and merged images, respectively. (**A-a3**) Control without liposomes, only medium; (**B-b3**) Unfunctionalized liposomes; (**C-c3**) Liposomes functionalized with gH625. The images indicate indicating no significant difference between the Lipo and gH625_Lipo groups compared to control. Scale bar corresponds to 10 µm.

8.5 Evaluation of gH625-liposome *in vivo*

8.5.1 Body temperature in mice

When injected intravenously in Swiss CD1 mice at the initial dose of 3 mg/kg Eq. NT, the gH625-liposomal formulation loaded with VH-N439 showed a strong hypothermic effect that was not significantly different from that observed with the free VH-N439. This result suggests that the encapsulation of VH-N439 in gH625-liposomes doesn't change the effect of free VH-N439. Moreover, the results showed that the hypothermic effect obtained with gH625-liposomes is significantly higher than that obtained without the gH625 peptide (**Figure 30 A**), demonstrating that gH625 may improve the BBB-crossing of liposomes and brain delivery of VH-N439. The results at 6 mg/kg Eq. NT showed a higher hypothermic effect of both liposomal formulations loaded with VH-N439 than that observed at 3 mg/kg Eq. NT, demonstrating a dose-response effect for both classes of liposomes loaded VH-N439. At this dose-level, gH625-functionalized liposomes still showed a significantly higher hypothermic effect than non-

functionalized liposomes (**Figure 30 B**). gH625-liposomes used as control showed no effect on body temperature.

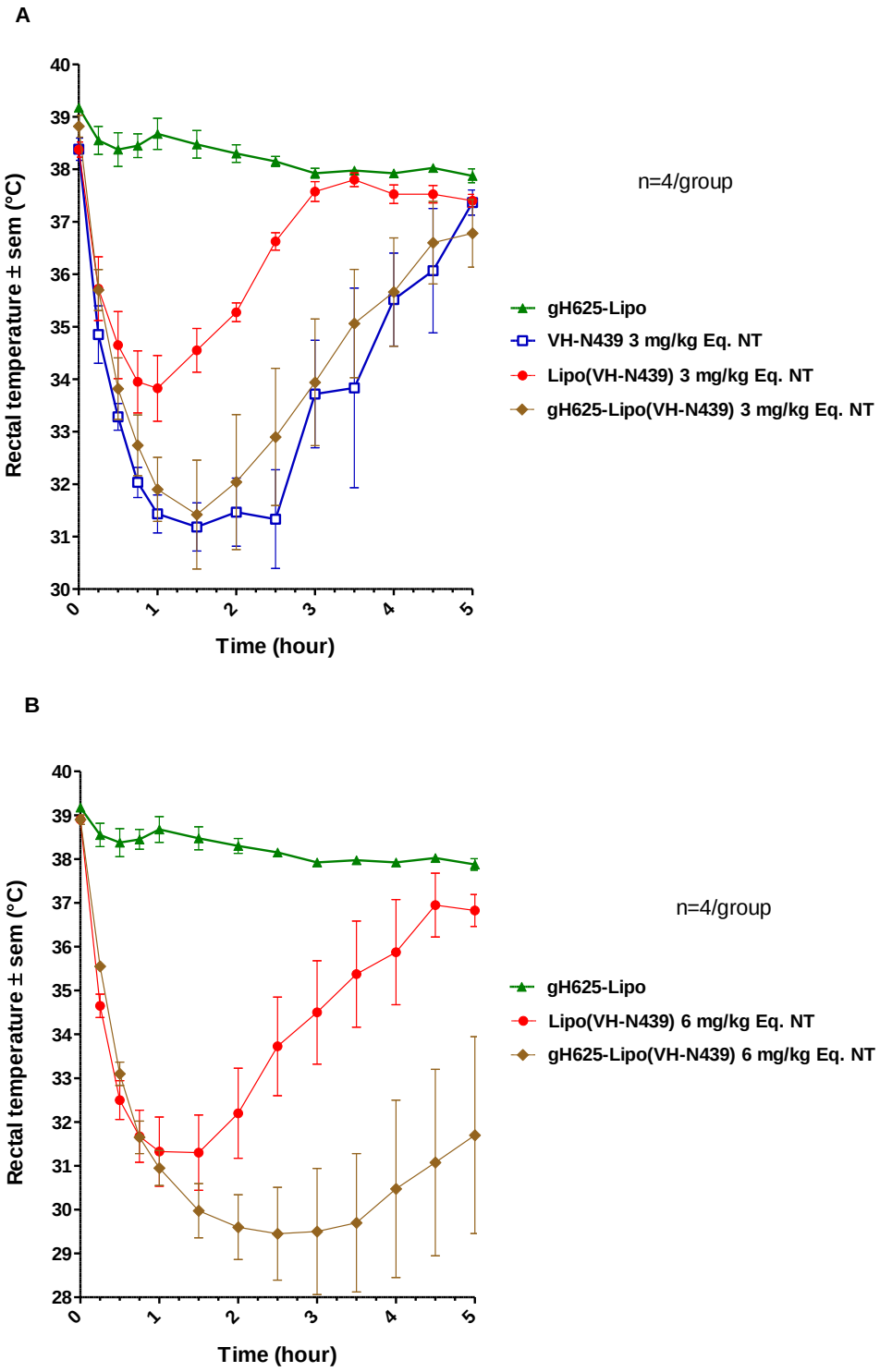

Figure 30: Effect of VH-N439 on body temperature in mice. gH625-Lipo(VH-N439) showed a strong hypothermic effect not significantly different from free VH-N439 and significantly higher than non-functionalized liposomes (**A**). The results at 6 mg/kg Eq. NT show a dose-response effect for both classes of liposomes loaded VH-N439 and significantly higher hypothermic effect for gH625-liposomes than non-functionalized liposomes (**B**).

9. DISCUSSION

The central nervous system (CNS), the most sensitive and critical organ system in the human body, is protected by the blood-brain barrier (BBB). The BBB ensures highly selective and efficient mechanisms to transport nutrients, regulate ion balance and prevent access of potentially toxic substances into the brain (Sanchez-Covarrubias et al., 2014). However, the BBB also significantly restricts the delivery of therapeutic molecules to the brain, thus making ineffective the treatment of several neurological diseases (Dominguez 2014). Despite numerous advances, pharmacological treatment of neurological disorders represents still a challenge for scientific community and less than 2% of all US Food and Drug Agency (FDA)-approved small-molecule drugs cross the intact BBB to varying degrees. During the last decade, drug delivery into the brain has been extensively investigated and several therapeutic approaches have been developed. These are mainly invasive approaches, such as direct intraventricular administration of drugs by means of surgery and temporary disruption of the BBB via intracarotid infusion of hyperosmolar solutions. Furthermore, noninvasive techniques have been also proposed including pharmacologically-based strategies which increase the lipid solubility of the drug to facilitate its passage across the BBB and/or exploit various carrier mechanisms (Lu et al., 2014). With the advent of nanomedicine, in the recent years, nanosystems have been proposed as noninvasive methods to cross the BBB and deliver drug to CNS (Holmes 2013; Re et al., 2012). Several nanocarriers have shown moderate success, mainly due to their behavior *in vivo* (Moros et al., 2013). Many of these carriers can be transported across *in vitro* and *in vivo* BBB models by endocytosis and/or transcytosis (Provenzale et al., 2005; Kreuter 2004; Jallouli et al., 2007) and have demonstrated preclinical success for treatment of CNS disorders (Wong et al., 2012). Among these the liposomes, lipid vesicles formed by one or more phospholipid bilayers and constituted of an aqueous core, have already had a major impact on many biomedical areas as drug delivery systems. Several liposomes have been approved for treatment of different pathologies while others are in clinical trials or are on the market (Bozzuto and Molinari 2015). They can transport hydrophilic drugs by encapsulating them in the aqueous core and amphiphilic and lipophilic drugs by solubilizing them in the phospholipid bilayer. Liposomes have as advantage to be easy to prepare, biocompatible, less toxic, and commercially available (Bhowmik et al., 2015). Recently, also peptide-based carriers have attracted considerable attention in the field of targeted drug delivery due to their high binding affinity and specificity for targets. The presence of advanced technologies for peptide identification and modification further supports the extensive application of peptides in the field of theranostics research (Doolittle et al., 2000; Hall et al., 2006). Short cationic and/or amphipathic peptides, known as cell-penetrating peptides (CPPs), have been used to mediate the drug delivery (Drin et al., 2003; Farkhani et al., 2014; Wang et al., 2014; Capolovici et al., 2014). They are able to transport several types of macromolecules across the membrane bilayer *in vitro* and *in vivo*

including small molecules, proteins, DNA/RNA, liposomes, peptides, and other supramolecular aggregates (Heitz et al., 2009; Vivès et al., 1997; Angeles-Boza et al., 2010). Although their mechanism of uptake is not still known, the endocytosis seems the main pathway involved (Jones 2008). The endocytosis may represent a problem; the cargo may be trapped in endosomes, eventually ending in lysosomes, by decreasing its intracellular bioavailability. Membranotropic CPPs represent particularly attractive tools for drug delivery. Their direct translocation across membranes could be immediately available in the cytosol the cargo transported, thus avoiding the risk of endosomal entrapment and degradation (Galdiero et al., 2013). gH625, a 20-residue peptide, is a membrane-perturbing domain that interacts with model membranes, contributing to their merging (Galdiero et al., 2010) and is able to traverse the membrane bilayer and transport a cargo (Falanga et al., 2011; Tarallo et al., 2011; Carberry et al., 2012) into the cytoplasm and across an *in vitro model* of the BBB (Guarnieri et al., 2013). Uptake studies suggested a nonactive translocation mechanism in crossing the lipid bilayer, which may vary depending on the cargo (Galdiero et al., 2015). Penetration by gH625 occurs in a rapid, concentration-dependent fashion that appears to be independent of receptors and transporters and instead is thought to target the lipid bilayer component of the cell membrane. Furthermore, many studies have been demonstrated that gH625 is able to deliver cargoes in several *in vitro* cell lines (Galdiero et al., 2013, 2014; Vitiello et al., 2011). Moreover, it has shown that decoration of NPs with gH625 significantly reduce cytotoxicity compared to blank-NPs, likely due to the ability of gH625 to avoid accumulation into lysosomes (Guarnieri et al., 2015). Although several studies have demonstrated that gH625 is an efficient carrier for bioactive cargoes *in vitro* (Falanga et al., 2013), these results do not guarantee that gH625 can be developed into a useful pharmaceutical delivery platform. Before any potential application, as for any potential pharmacological approach, it is necessary to translate *in vitro* findings to animal models to determine whether gH625 is able to deliver molecules into cells of organisms and to keep track of the whole biological picture and the complex nano-biological interactions. On the basis of this interesting background, the aim of this Ph.D. project was to evaluate if gH625 can be used to develop efficient nanocarriers to deliver therapeutic agents to the brain. *In vitro* studies showed that both human neuroblastoma (SH-SY5Y) and glioblastoma-astrocytoma cell lines (U-87 MG) can take up more than 90% of administered gH625, indicating that internalization of gH625 into these cell lines is an efficient process. Uptake of the gH625 was not associated with variation in cell viability, indicating that the peptide was devoid of cytotoxic effects at the dose tested.

The results of *in vivo* administration of gH625 in rats demonstrated that, despite the blood filtration action of the liver, carried out through the gH625 uptake of Kupffer cells, gH625 substantially reaches the brain BBB vessels. Interestingly, gH625 could be found in the BBB endothelium, so this peptide can be efficiently accumulated in endothelial cells of the BBB. It was also evaluated whether gH625 had actually crossed the BBB or was simply trapped within

endothelial cells of the brain. In particular, although was not detected any association with astrocytes, gH625 was found in some neurons, suggesting that gH625 reaches the cortical perikarya and their processes. The presence of multiple metabolic barriers may restrict the application of such a peptide-based ligand for targeted drug delivery *in vivo*. Peptides alone or conjugated on the surface of nanocarriers are subject to proteolysis in the blood after systemic administration. In addition, the BBB is also a metabolic barrier due to the presence of various enzymes in brain capillary endothelial cells. However, despite these limitations, the results of this study demonstrated that gH625 intact peptide was still present after 3.5 hours from intravenous administration in rat, and thus it has potential for extending brain targeting efficiency due to its resistance to proteolysis for 3.5 hours.

On the basis of these results, it was also tested the efficiency of liposomes functionalized with gH625 to transport a neuroprotective peptide through the endothelium of an *in vitro* model of rat BBB. Liposomes were charged with PACAP (pituitary adenylate cyclase-activating polypeptide), a neurotrophic and neuroprotective peptide proposed for treatment of CNS injuries, stroke, and neurodegenerative diseases (Lee and Seo 2014), which shows a rapid degradation in the blood and limited accumulation in the brain (Meredith et al., 2015). The results showed that the functionalization of liposomes with gH625 improves their passage through the endothelium of *in vitro* BBB model, thus resulting in an increased transport of PACAP and its less accumulation in the endothelial cells. In particular, the amount of PACAP which cross the BBB monolayer after 30 minutes of incubation is 19.4% higher when transported by gH625-liposomes than unfunctionalized liposomes and 44.3% higher after 60 minutes. Furthermore, the amount of PACAP transported across the BBB monolayer by gH625-Liposomes increases in the time, of 29% from 30 minutes to 60 minutes and of 36,5% from 60 minutes to 120 minutes. In addition, the gH625-liposomes are nontoxic and do not affect tight junction organization in the BBB endothelium.

The *in vivo* experiments in mice using VH-N439 showed that the gH625-liposomal formulation loaded with VH-N439 showed a strong hypothermic effect that was not significantly different from that observed with the free VH-N439 and significantly higher than that obtained without the gH625 peptide. This result demonstrates that gH625 improves the brain delivery of liposomes. Moreover both liposomal formulations loaded with VH-N439 showed a dose-response effect.

Although the exact molecular mechanism for the entry of gH625 remains to be established, the results of this study show for the first time that a membranotropic peptide derived from *Herpes Simplex virus* type 1 can be incorporated *in vitro* by neuron or astrocyte cells, has the ability to accumulate *in vivo* in the brain and improves the passage of liposomes through the endothelium of a BBB *in vitro* model. The fact that it is a membranotropic peptide able to interact with the membrane bilayer and destabilize it locally and temporarily, suggests translocation across the membrane bilayer that does not seem to involve classical endocytosis mechanisms and,

consequently, the cargo may be immediate bioavailable (Galdiero et al., 2014; Guarnieri et al., 2013). Due its features gH625 could transport a wide variety of cargoes with intact bioactivity into almost any tissue or organ, resulting no specific for the brain. However, nonselective transduction is not a drawback because specific targeting units may be added in the delivery system to increase retention in specific tissues. In particular, liposomes result very interesting for this approach. In fact, the liposomes surface may be easily decorated with different ligands such as antibodies, peptides/proteins and carbohydrates (Sercombe et al., 2015). Liposome modified with gH625 and a ligand for BBB may be promising tool as drug delivery system for CNS, using gH625 to increase the transport across the endothelium of BBB and a specific ligand to improve the target for brain. This approach could open the way to deliver and upgrade many neuroprotective and neuroriparative agents which have limitation to use such as low bioavaibility, toxicity, or impermability to BBB. Taken together, these data suggest that gH625 peptide could be used as a brain delivery system for macromolecules and it is a useful tool to develop nanosystems gH625-mediated to deliver therapeutic agents to CNS. Further experiments *in vivo* are underway with various therapeutic targets, including small unstable molecules, macromolecules, and other pharmaceutical agents in order to evaluate if gH625 can be developed into a useful pharmaceutical delivery platform. This Ph.D. project demonstrated that gH625-liposomes as a promising carrier system to deliver therapeutic molecules through the BBB.

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