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Jumping hurdles: Peptides able to overcome biological barriers

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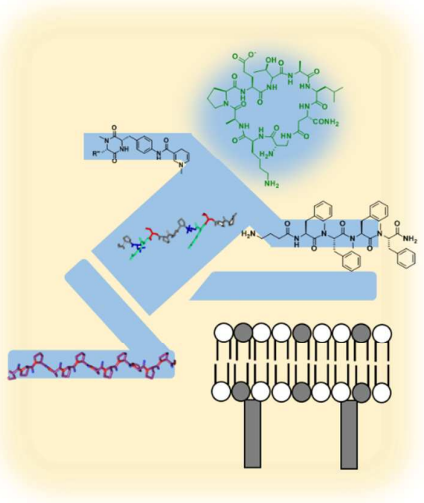

The cell membrane, the gastrointestinal tract or the blood-brain barrier (BBB) are good examples of biological barriers that define and protect cells and organs. They impose different level of restrictions but they also share common features. For instance, they all display a high lipophilic character. For this reason, hydrophilic compounds, like peptides, proteins or nucleic acids have long been considered as unable to bypass them. However, the discovery of cell-penetrating peptides (CPPs), opened a vast field of research. Nowadays, CPPs, homing peptides and blood-brain barrier peptide shuttles (BBB-shuttles) are good examples of peptides able to target and/or to cross various biological barriers.

CPPs are a group of peptides able to interact with the plasma membrane and enter the cell. The display some common characteristics like positive charged residues, mainly arginines, and amphipathicity. In this field, our group has been focused on the development of proline rich CPPs and in the analysis of the importance of secondary amphipathicity in the internalization process. Proline has a privileged structure being the only aminoacid with a secondary amine and a cyclic side chain. These features constrain its structure and hamper the formation of H-bond. Taking advantage of this privileged structure, three different families of proline-rich peptides have been developed, namely, proline-rich dendrimer, sweet arrow peptide (SAP) and a group of foldamers based on γ-peptides. The structure and the mechanism of internalization of all of them has been evaluated and analyzed.

BBB-shuttles are peptides able to cross the BBB and to carry with them compounds that cannot reach the brain parenchyma unaided. These peptides take advantage of the natural transport mechanisms present at the BBB, which are divided in active and passive transport mechanisms. On the one hand, we have developed BBB-shuttles that cross the BBB by passive transport mechanism, like diketoperazines (DKPs), $(N\text{-MePhe})_n$ or $(PhPro)_n$. On the other hand, we have investigated BBB-shuttles that utilizes active transport mechanism such as SGV, THRre or MiniAp-4. For the development of both groups we have explored several approaches, such as the use of peptide libraries, both chemical and from phage display, or hit-to-lead optimization processes.

In this article, we describe, by chronologic order, our contribution to the development of peptides able to overcome various biological barriers and our efforts in order to understand the mechanism that they display. In addition, the potential use of both, CPP and BBB-shuttles, to improve the transport of promising therapeutic compounds is described.

Introduction

Delivery of bioactive compounds through various biological barriers has been a challenge for a long period of time. The plasma membrane, the gastrointestinal barrier or the blood-brain barrier (BBB) are examples of biological hurdles that limit the access of therapeutics to the desired target. The simplest of them, the cellular membrane, has

being traditionally considered impermeable to hydrophilic compounds since only lipophilic compounds were able to interact with the lipid bilayer. Thus, delivery of peptides, proteins or nucleic acids to the cytoplasm was considered a utopia thirty years ago. However, the discovery of penetratin and TAT peptides, based on natural proteins, opened a vast field of research.[1,2] This new family of compounds, named cell-penetrating peptides (CPPs) accounts nowadays for more than 100 members, with various origins.[3] During its development several challenges have been addressed, such as endosomal entrapment upon internalization[4] or protease liability.[5] To date, CPPs have been used to deliver several cargoes into the cell, such as nanoparticles of various types, nucleic acids or proteins.[6]

Intracellular targets are now readily accessible by using CPPs but there are other biological barriers that preclude the access of drugs to their target. For instance, oral delivery is hampered by the gastrointestinal barrier.[7] More difficult to overcome is the BBB that limits the access of 98% of drugs to the brain while completely preventing the passage of macromolecules.[8] In both cases, several peptides have been described to interact with some of the components of these barriers with the aim to overcome them.[9-11]

During this process of evolution our group have played an active role in the development and study of CPPs, first, and then brain delivery vectors. The purpose of this Account is to give an overview on peptides able to overcome biological barriers with a special focus on recent work from our group.

Proline rich cell-penetrating peptides

CPPs can be classified according to various parameters, such as sequence or internalization mechanism, to name a few.[3] The latter is affected by several factors, such as net charge or structure. For instance, amphipathicity, imposed by the sequence of the peptide, and secondary amphipathicity, determined by peptide secondary structure, are key for internalization. Amphipathic alpha helixes, defined by having two separated hydrophobic and hydrophilic faces, are a shared structural motif in a wide variety of CPPs.[12]

Proline is one of the 20 genetically encoded amino acids but the only one with a secondary amine and a cyclic side chain which constrain its structure. Moreover, the amino group of proline cannot participate in H-bond, unlike in the other natural amino acids. These characteristics confer unique structural properties to polyproline peptides which adopt well defined conformations that vary depending on the environment (Figure 1A). In organic solvents, polyproline adopts a polyproline I (PPI) conformation, which is characterized by being a right handed *cis* orientated helix with 1.9 residues per turn. In aqueous solutions, polyproline adopts a PPII conformation, meaning a left handed helix, *trans* oriented and with 3.0 residues per turn.[13] These particular secondary structures inspired us to design a drug delivery system consisting of proline based dendrimers (Figure 1D).[14] Peptide based dendrimers are unique compounds.[15] They can be seen as globular proteins and among all the potential applications, their branched structure can facilitate drug entrapment. We proposed that this property would be stressed in our system due to the plasticity conferred by the polyproline sequence. The dendrimer was prepared using *cis*-4 amino-L-proline as the scaffold (Figure 1C).[16,17] The second amino group was used as a branch point. Extensive conformational analysis

proved the transition from a compacted PPI structure to a more extended PPII conformation as well as the contrary. The antibiotic ciprofloxacin was successfully encapsulated. The potential of this system was further analysed by evaluating its internalization properties. Interestingly, the prepared dendrimer internalized in rat kidney cells but, surprisingly, the control peptide (P_{14}) did as well.[14] This result, together with our experience with γ-zein protein, led us to study the internalization capabilities of $(VXLPPP)_n$ peptides. The amphipathic secondary structure but non amphipathic sequence make these peptides very facinating. We prepared a library of (VXLPPP)n (X= H, K, R; n= 1,2,3) and compared to P_m (m= 6,12,18). The peptide with better internalization properties on HeLa was $(VRLPPP)_3$ (Figure 1B).[18] Remarkably, when compared with TAT and penetratin, the new designed vectors displayed no toxicity despite of the internalization differences. The interesting internalization properties of $(VRLPPP)_3$, named the sweet arrow peptide (SAP), have been enhanced by introducing fatty acid moieties on its sequence[19] or by mutating one of the proline residues to silaproline.[20] Noteworthy, none of the aforementioned SAP versions displayed cytotoxicity that combined with the non-viral origin and high solubility of these compounds make them very interesting CPPs.

CPP internalization mechanism has generated some debate in the scientific community.[21] Discussion around the existence or not of an endocytic pathway has promoted several studies. In this context, we analysed the internalization mechanism of SAP finding evidence of lipid raft mediated mechanism.[22] Analysis of the intracellular pathway followed by SAP combined with the stability in front of proteases suggested endosomal release of the peptide to the cytoplasm rather than degradation.

Liability to proteases is one of the main drawbacks of using peptides as therapeutics and it can be consider as another biological barrier to overcome. Among the several strategies that can be applied to enhance this stability the use of D-amino acids stands out. SAP has good stability versus proteases but for further applications we explored the synthesis of a fully protease resistant version by preparing the peptide with D-amino acids (Figure 1B).[23] $(vrlppp)_3$ was shown to be remarkably more stable than its counterpart, both in serum and in front of trypsin. Remarkably, analysis of the internalization mechanism showed that both versions behaved similarly suggesting that the internalization process was receptor independent since the stereochemistry of the α-carbon does not affect. Further analysis, by circular dichroism (CD) showed that both peptides had a PPII structure but inverted. The peptide kept the propensity to form fibrillary aggregates, demonstrating that neither the inversion of all the α-carbon stereogenic centers nor the consequent change in helix handedness affect to the overall topology of the aggregate. Importantly, the new version, preserved the same CPP properties than its L-counterpart. Heparin competition assays showed minor impact in internalization ruling out electrostatic interactions with the glycocalix as a main internalization trigger. Further studies confirmed an active internalization pathway. Again, lipid raft/caveolae was seen as the most plausible internalization mechanism. Preliminary studies in mice proved the suitability of D-SAP for *in vivo* applications.[24]

Taking into account the lack of inhibition of the internalization of SAP by heparin and in order of fully validate the importance of the PPII configuration in the internalization process, a negative version of SAP, SAP(E), was prepared, by mutating arginine to

glutamic acid (Figure 1B).[25] The internalization mechanism of this new peptide was confirmed as the same for SAP, opening the venue for delivering more challenging cargoes. Most of the CPP described to date are either cationic or amphipathic, being optimal for complexation with DNA and other negatively charged molecules. A negative charged CPP would allow for complexation to yet unexplored cargoes with positive net charge. Interestingly, both SAP and SAP(E) have been recently used to deliver SiC-NP to cells.[26]

All these results suggest that internalization of SAP and SAP(E) may be triggered or affected by its self-assembly.[27] Recent analyses of the interaction of SAP(E) with lipids, as a model of the cytoplasmic membrane, demonstrate that only interaction with the polar head groups occur and no membrane insertion is observed.[28]

Our interest in proline based structures lead us to develop a new family of CPPs consisting on γ-peptides, using *cis*-4-amino-L-proline as a scaffold. In this family of foldamers, the peptide amide bond was formed at the amino group at position 4, and the α-amino group was used to introduce different groups, both acyl and alkyl chains (Figure 1E). Exhaustive analysis by both NMR and CD allowed for the identification of secondary structures suggesting the formation of isolated H-bond ribbon, identifying the smallest β-strand.[29] Evaluation of the internalization mechanism of a selection of these peptides suggested an endocytic pathway. This family of peptides displays improved solubility, low cytotoxicity, remarkably stability versus proteases and good internalization properties.[30]

Brain targeting peptide BBB-shuttles

CPPs enter the cell by a not yet completely understood mechanism. What is clear is that tissue selectivity is not always achieved. Some peptides, known as homing peptides, are designed to recognize specific tissues.[31] However, in the case of the brain, this selective transport is even more difficult to get due to the presence of the BBB. The BBB is a physiological and metabolic barrier resulting from a complex interaction between endothelial cells and several cells types, like pericytes or end foot astrocytes present at the brain. The main function of the BBB is to protect the brain limiting the entrance of unwanted substances. Several restrictions like low vesicular transport, the presence of tight junctions, a high proteolytic activity or the presence of efflux pumps limit the access of exogenous substances to the brain. These features make delivery of therapeutics difficult. An early work in 1999, showed that a 120 kDa enzyme conjugated to TAT was delivered in its active form into the brain.[32] Despite of the lack of brain selectivity, this seminal study paved the way for peptide mediated brain delivery. Initial studies evaluated some of the most promising CPPs as brain delivery vectors. Although brain penetration was accomplished in some cases, selectivity was a major problem. Moreover, poor analysis methods make difficult to distinguish among parenchyma distribution of the peptide and entrapment in the brain capillaries.[10] A recent study has demonstrated that CPPs can enter the brain but their efflux rate is greater than their influx one, limiting its use as brain delivery vectors.[33]

The brain has high energy requirements having several transport mechanisms that could be ideally exploited for brain delivery. For instance, small molecules like glucose or amino acids have specific transporters and proteins like transferrin or insulin have

specific receptors. Additionally, unspecific transport mechanisms can be observed as well, such us passive diffusion or adsorptive-mediated transcytosis. The various transport mechanisms can be classified into passive or active.[10]

Passive crossing

Numerous strategies have been developed to improve brain uptake of therapeutics.[34] Among them, we have focused on the development of specific peptide brain delivery vectors, named BBB-shuttles.[10] One of the first approaches that we explored consisted on design of BBB-shuttles by using a genetic algorithm which optimized the physico-chemical properties of the peptides in order to increase their passive transport.[35] The optimization process consisted in reduction of hydrogen bonding units on the peptide, fine tuning of the logP and increase of lipophilicity, among others. It yielded a set of peptides with several *N*-methylations that complicated the synthesis of the library.[36] Analysis of the library led to the discovery of two candidates to BBB-shuttle highlighting the potential of genetic algorithms to generate new brain delivery vectors.[37]

During the preparation of the previous library, diketopiperazine (DKP) formation was observed (Figure 2A). Interestingly, one of the subproducts, DKP *N*-MePhe-*N*-MePhe, had very good transport through the tested methods. This result motivated us to prepare two libraries of DKPs, mono and di-*N*-methylated in order to assess if the second methyl group improved the transport or increased membrane retention. This was the case for the highly hydrophobic DKPs, whilst most of them showed higher permeability. Moreover, two relevant cargoes were evaluated, baicalin, a flavonoid that inhibits prolyloligopeptidase (POP), and L-DOPA, a precursor of dopamine, improving the in both cases, the permeability of the cargo. In the case of baicalin, its POP inhibitory activity was also preserved.[38] The application of DKPs was further explored in the design of chemical delivery systems which consisted of brain permeable compounds that would undergo chemical transformation once in the brain parenchyma (Figure 2A'). This transformation would trap the compound inside the brain leading to a sustained delivery of the compound of interest. Introduction of *N*-methyldihydropyridine moiety to the DKP scaffold conferred the desired reactivity to the systems. This system has been validated for L-DOPA.[39] Evaluation of both, permeability through the BBB, by means of parallel artificial membrane permeability assay (PAMPA), and POP inhibition confirmed our hypothesis.[40] DKPs have recently been evalutated *in vivo*[41] and for dermal delivery of small drugs.[42]

The use of peptide libraries is a very attractive tool to discover new hits with a given activity. We have used several types of libraries to find shuttles targeting BBB transport mechanism. For instance, considering the potential of passive diffusion shuttles to increase the brain uptake of small polar drugs, we have investigated various libraries of peptides designed to passively cross the BBB.[43-46] Molecular weight, lipophilicity and hydrogen bonding are the key parameters to study. Based on our experience on the use of *N*-methyl amino acids[36] we selected this feature as key parameter of some of the libraries created. In a first work, *N*-methylphenylalanine (*N*-MePhe) (Figure 2B), a fairly common amino acid in nature, was selected as a monomer to create a library of potential BBB-shuttles.[47] First, the length of the peptides was adjusted with 4 amino acids being the optimal length.[46] Then, the *C* and *N* terminus were optimized to acetyl

and amide group, respectively. At this point, the fourth amino acid was mutated. The peptides with *N*-MePhe, cyclohexylalanine (Cha), and 2-naphtylalanine (2Nal) had the higher transport. These 3 peptides were selected to transport L-DOPA, enhancing its permeability. Noteworthy, all the peptides have remarkable stability in front of serum proteases. Later, the applicability of these shuttles was expanded to other cargoes.[45] Comparison to a library of proline based peptides pointed to the phenyl group as responsible for the privileged transport.[46]

Although the concept of an universal shuttle is very attractive, several studies indicate that the right approach contemplates a *one shuttle-one cargo* strategy.[10] Knowledge about the key parameters that influence BBB transport can help to fine tune a given shuttle. Exploiting this theory we have evaluated several parameters to create BBB-shuttles at will.[44] The first parameter to evaluate was the chirality. Based on *N*-MePhe, a library of 16 BBB-shuttles was prepared, combining all diastereoisomers. PAMPA and artificial membrane chromatography (IAMC) were used as evaluation tools. Various conclusions were extracted. First, homochiral peptides (DDDD or LLLL) showed less lipophilicity and higher permeability. Second, when increasing the lipophilicity, the membrane retention was higher resulting in a diminution of the permeability. Third, enantiomeric discrimination was observed. With these results in hand, we explored if the permeability of a cargo can be fine-tuned by modifying the selected shuttle. With this aim, three different cargoes were compared (Figure 2c). For two of them, the tetramer gave better results while for one the trimer was the most efficient. With this result an *a la carte* shuttle is described (Figure 2C). After, other parameters such as non-natural amino acid incorporation and halogenation were analysed. In all cases, cargo-based differences were found. Several factors such as peptide shuttle length, stereochemistry or halogen content can be fine-tuned to fully optimize the transport of the drug of interest.[44]

Despite of the promising features of the described BBB-shuttles, their reduced water solubility (<1μM) can hamper its use. With the aim of developing water soluble shuttles we decided to prepare a library of potential shuttles by combining the characteristics of *N*-MePhe and polyproline peptides, by preparing polyphenylproline peptides. Proline has a restricted conformation due to its cyclic side chain and high solubility in water. Moreover, the phenyl group has been reported to have a positive impact on membrane penetration.[25] To merge the advantages of both amino acids, *cis*-3-phenylpyrrolidine-2-carboxilic acid (PhPro) (Figure 2B) was used as scaffold. When comparing the diastereomeric mixture of $(N\text{-MePhe})_4$ to $(\text{PhPro})_4$, a similar transport profile was observed by PAMPA. Moreover, the transport of two cargoes further demonstrated the shuttle capability of this new family of compounds. Noteworthy, the water solubility was increased 1000-fold. In two pairs of enantiomers was enantiomeric discrimination observed. These peptides, displayed a complete different structure from the one of the parent peptides (Pro_4 and $N\text{-MePhe}_4$) suggesting the new secondary structure as main reason of the discrimination observed at the membrane. This work provides a new parameter, enantiomeric discrimination, to evaluate when designing peptides to cross biological membranes by passive diffusion mechanism.[43]

Active crossing

Among the several transport mechanism available at the BBB, active transport mechanism offers several advantages. By targeting specific receptors, the delivery to peripheral tissues will be reduced. Additionally, it allows for the transport of a wider range of compounds that can vary in size, charge and nature.

In order to discover new BBB-shuttles that target an active transport mechanism, a phage display (PD) peptide library was confronted to an *in vitro* human cell-based BBB model (Figure 3),[48] leading to the discovery of SGV. The transport of this peptide was corroborated in the same assay obtaining permeability values similar to previous described shuttles.[49,50] This work validated the use of PD against human cell-based models of the BBB as a valuable strategy to discover new BBB-shuttles.

The main limitation of PD techniques is that the peptides obtained are formed by natural amino acids, thus being labile to proteases. To solve this problem, we have generated peptide libraries using non-natural amino acids. For instance, we designed a chemical library using one-bead-one-compound strategy. For that, a selection of 6 D versions of natural amino acids that covered the chemical space and a mix-and-split technique were used to generate a library of fully protease resistant heptapeptides,[51] ensuring the preparation of a highly heterogeneous library. The selection of the amino acids was done after careful analysis considering several parameters such as the preference of the amino acid to participate in protein-protein interaction or the pKa at physiological pH, to name a few. Combination with *in vitro* models, that mimicked the BBB, and *state-of-the-art* mass spectrometry led to the discovery of new BBB-shuttles. The first step was the validation of the quality of the library. Then, two BBB models were used to extract the sequence of interest. An *in vitro* cell-based model was used in combination with PAMPA to discriminate among active and passive transported shuttles. This process was followed by two levels of exhaustive MS analysis, to identify the peptide family and the specific peptide. In this article, a stimulating way of obtaining active compounds by combining combinatorial chemistry with advanced, but available, MS analysis was described.

Within this last section, we have described our efforts in the discovery of new BBB-shuttles, mainly by using libraries, both chemical and based on PD. However, other strategies have been explored as sources of BBB-shuttles. Two last examples of active transported BBB-shuttle have been discovered by hit-to-lead optimizations of given BBB-shuttle candidates.[49,50]

The first example is THRre,[49] a peptide fully resistant to proteases discovered by applying the retro-enantio approach to THR.[52] THR was discovered by PD against the human transferrin receptor (hTfR).[53] This receptor is highly expressed at the BBB and could be envisaged as a gate of entrance for therapeutics. To test this hypothesis we prepared gold nanoparticles (AuNP) modified with THR and CLPFFD, an anti-amyloid peptide. Increase in BBB transport of THR modified AuNP was observed both *in vitro* and *in vivo*. However, the most important finding was the detection of modified nanoparticles at various sections of the mouse brain parenchyma, unequivocally proving BBB passage of the AuNP system (Figure 4A).[52] Up to 0.16 ± 0.11 μg of gold/brain (dry tissue) which correspond to a 0.0765 % of the injected dose was found. This discovery lead us to further improve the properties of THR. THRre maintain some of

the topological features of THR, since it was prepared by reversing the amino acid sequence and changing the stereochemistry of the side chains. Several internalization studies confirmed TfR as the entry mechanism and showed no competition with transferrin. THRre was shown to transport a wide variety of cargoes. Interestingly, *in vivo* two-photon microscopy proved the delivery of THRre modified QDs to the brain parenchyma (Figure 4B). We are currently analysing the potential of this peptide.

The second example of hit-to-lead optimization process is MiniAp-4.[50] This time, we were inspired by the exquisite composition of venoms. Venoms are rich in peptides with important activity at very low doses. We hypothesised that venoms can be a source of BBB-shuttles if their toxicity would be reduced, since some of them affect the central nervous system (CNS). As a proof-of-concept, we studied apamin, the main component of bee venom that has been reported to have CNS activity.[54] Luckily, the residues involved in toxicity are known and preliminary studies showed that point mutations were sufficient to reduce the toxicity of the peptide (Figure 5A and B). By using an *in vitro* cell-based model we evaluated the transport of both peptides, apamin and its non-toxic analog, ApOO, finding evidence of an active transport mechanism. By this study, we suggest the use of modified versions of apamin as BBB-shuttles and point out venoms as its sources.[34]

Both Apamin and ApOO have promising characteristics as BBB-shuttles. However, their immunogenicity, together with the complex synthesis, hamper their use. In order to reduce the immunogenicity and the toxicity of these peptides while preserving the brain targeting capacity, various minimized versions of apamin were prepared. For the design, we selected the part of the sequence not involved in the toxicity, namely, the loop between cysteine 3 and 11. In order to preserve the protease stability, a non-natural linkage, lactam, was suggested as disulphide bridge surrogate (Figure 5C). The resulting macrocycle had improved transport in cell-based BBB model when compared to apamin (Figure 6).[50] Additionally, its ability to transport a wide variety of cargoes, from metallic nanoparticles to proteins, across endothelial cell monolayers, was validated. More importantly, we proved that the immunogenic and toxic properties of the apamin were abolished.

Conclusions

The potential of peptides in biomedicine applies to all the areas, from diagnostics to treatment. Peptides can modulate protein-protein interactions,[55] can be used as targeting moieties, etc. During the last 30 years, drug delivery has been one of the fields where peptides have caused a high impact, as proved by the development of CPPs, homing peptides and BBB-shuttles, amongst others.[9,10,21,31,56] Some of this new vectors have even reached clinical trials.[57] Our contribution to the field has been focused on the search on protease resistant peptides. The introduction of key features like *N*-methylation, D-amino acids or cyclisation, help us to modulate interactions of interest by reducing the number of H-bonds, the lipophilicity of the compounds or by fixing the conformation of the peptides.

Better knowledge of the composition of the various biological barriers will lead to an improved design of peptides able to overcome them. To get to this point, a synergistic

approach, which combines receptor analysis and membrane composition, with screening of targeted libraries may be needed.

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Biographical information

Macarena Sánchez Navarro obtained her PhD in 2009 for her work on the synthesis of glycodendrimers. In a first postdoctoral stay, she worked on the preparation and evaluation of two families of water soluble fullerenes. In 2010, she joined the University of Oxford where she was involved in the site-selective modification of proteins. Currently she is Research Associate at the group of Prof. Ernest Giralt at IRB Barcelona where her research focusses in understanding the main mechanisms of brain transport.

Meritxell Teixidó has been a Research Associate at the IRB Barcelona since 2006. Her major interests are peptide synthesis and discovery of blood–brain barrier peptide shuttles and its use to deliver drugs, diagnostic agents and nanoparticles that otherwise cannot reach their target inside the CNS. Her research combines protease-resistant peptides, mass spectrometry techniques, and transport evaluation tools to achieve delivery systems.

Ernest Giralt is Professor of Organic Chemistry at the University of Barcelona and Group Leader at the IRB Barcelona where he is the Head of the Chemistry and Molecular Pharmacology Programme. He has received several awards including, the Josef Rüdinger Memorial Lecture Award and the Max Bergmann Medal. His major interests lie in the fields of peptide synthesis and molecular recognition, in particular using NMR, with emphasis on the design of specific ligands for interaction with protein surfaces, related to possible therapeutic uses. This includes studies concerning new brain delivery systems, and modulators of protein–protein interactions.

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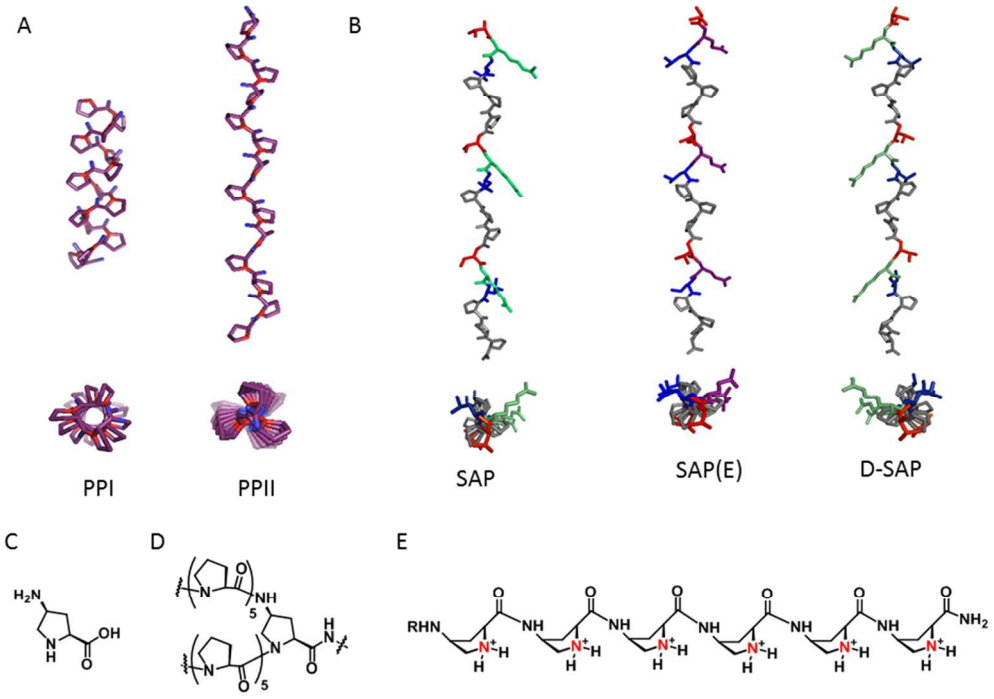

Figure 1. A) Structure of PPI and PPII; B) Structure of SAP, SAP(E) and D-SAP; C) cis-4-amino-L-proline; D) Core of polyproline dendrimer; E) γ-peptides based on cis-4-amino-L-proline.

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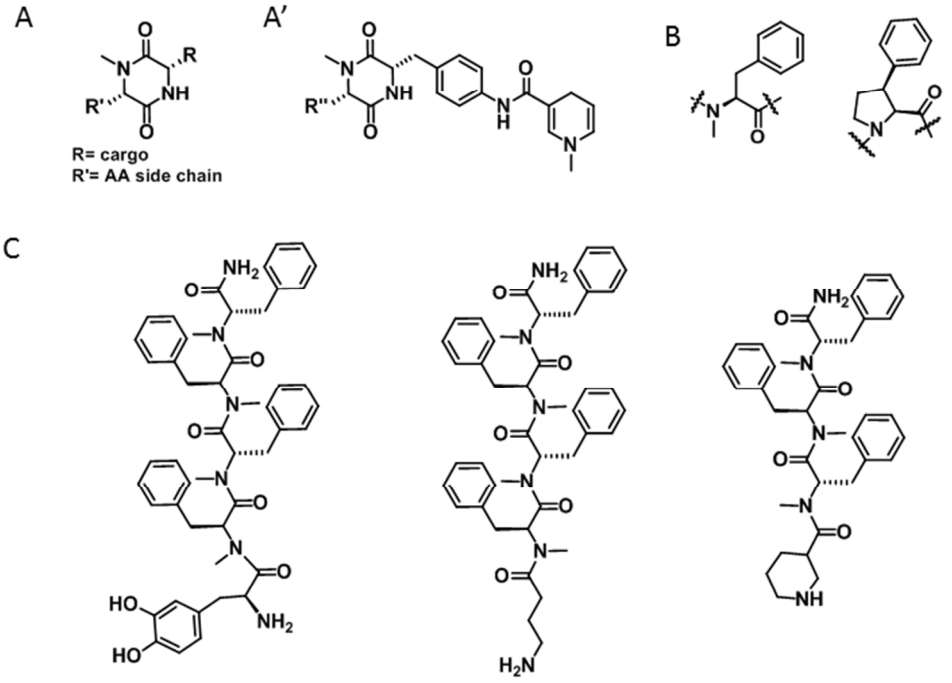

Figure 2. A) DKP with A') DKP-POP inhibitor; B) N-MePhe and PhPro; C) Example of a la carte shuttles for L-DOPA, levulinic and nipecotic acid, respectively.

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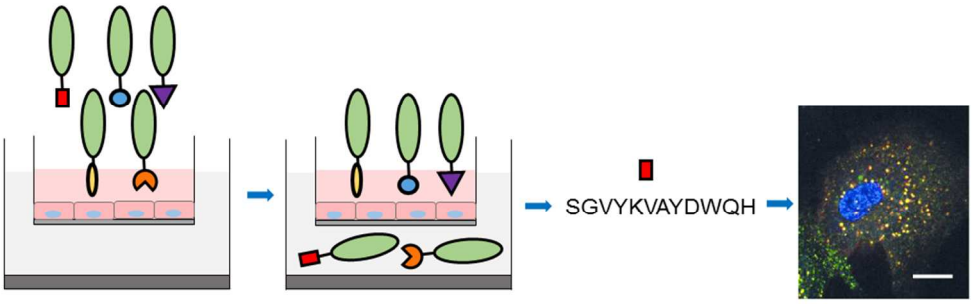

Figure 3. Schematic representation of the process of discovery of SGV: biopanning of a PD library against a human BBB cell-based model followed by in vitro evaluation of the selected sequence.

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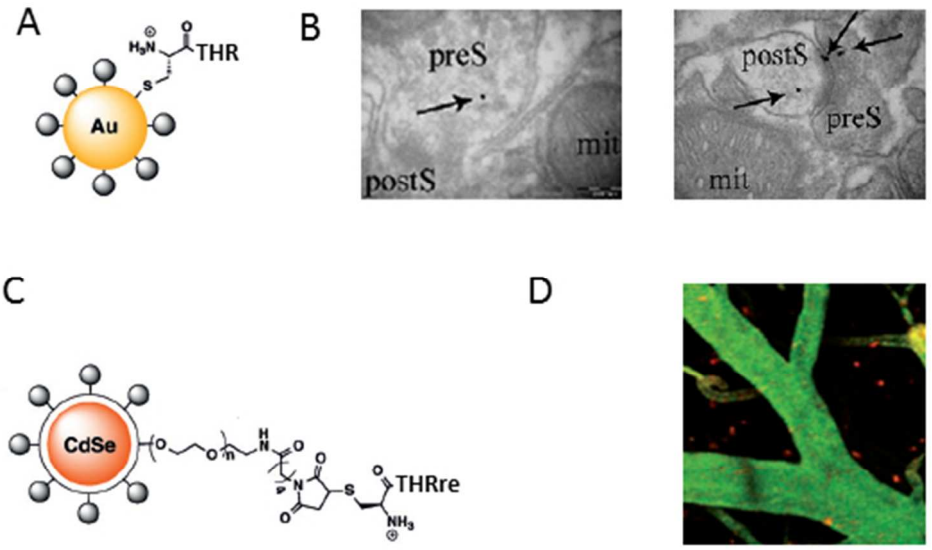

Figure 4. A) Schematic representation of AuNP modified with THR. B) TEM micrographs of mice brains injected with AuNP-THR.52 Arrows point to AuNP. C) Schematic representation of QDs modified with THRre. D) Intravital two-photon microscopy images of the brains of mice after injection of QDs-THRre (in red) capillaries are stained in green by FITC-dextran.

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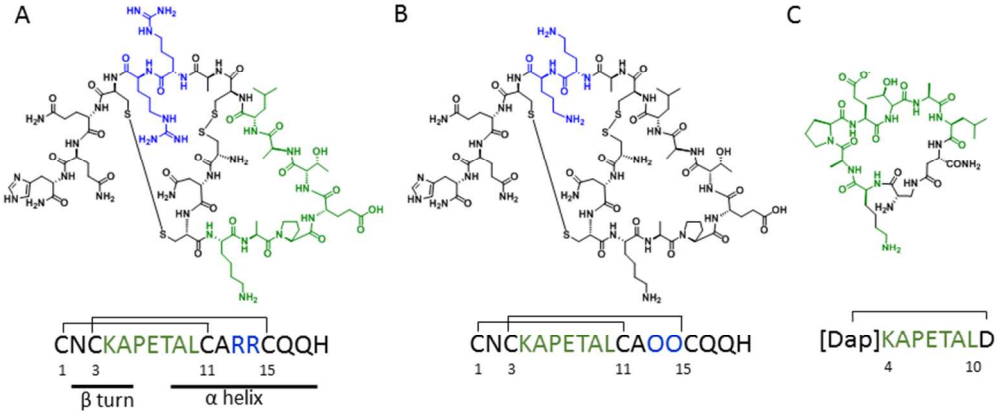

Figure 5. Apamine derived peptides. A) Apamin. B) ApOO C) MiniAp-4. Common residues of Apamine and MiniAp-4 are highlighted in green. Mutated residues from Apamin to ApOO are highlighted in blue.

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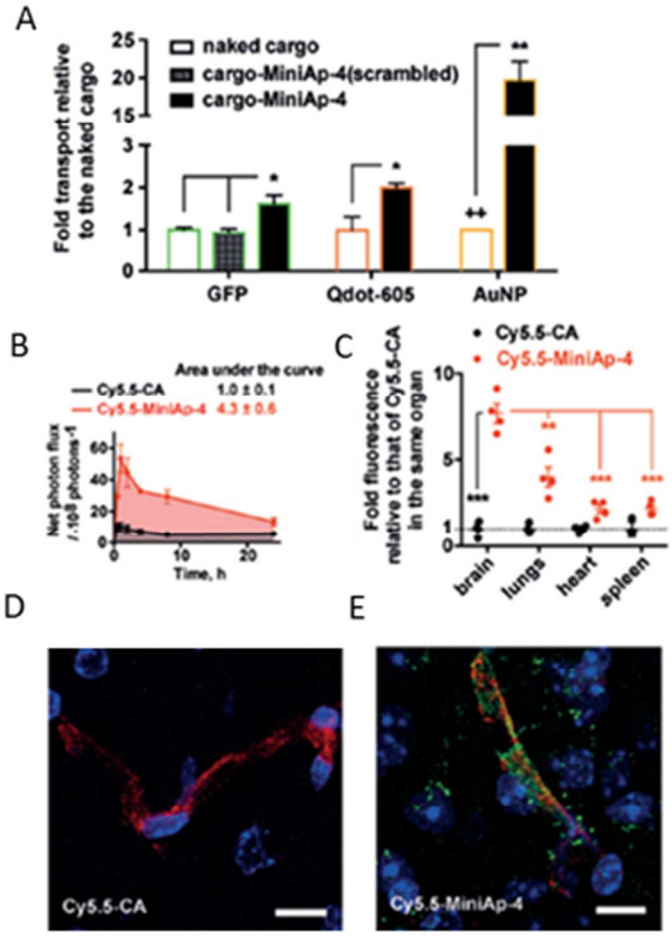

Figure 6. A) Increase in transport of MiniAp-4 modified cargoes in a human-cell-based BBB model. B) Fluorescence intensity of the brain region measured in vivo. C) Fluorescence intensity of various organs ex vivo. D) Representative confocal microscopy images of brain slices (cortex) of mice injected with Cy5.5–MiniAp-4 (top) and control (bottom). The Cy5.5 conjugates are shown in green, capillaries in red, and cell nuclei in blue. Scale bars: 10 mm. Error bars represent the SEM (n=3, *p<0.05, **p<0.01, ***p<0.001). + + indicates the quantification limit.

91x126mm (96 x 96 DPI)