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Delivery of Chemically Modified Peptides and Proteins through the Blood-Brain Barrier

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Abstract. Peptide- and protein-based signal substances serve important functions in our body. Neuropeptides are a class of neurotransmitters involved in specific communication in the peripheral and central nervous system. Therefore, natural and synthetic neuropeptides have potential significance as neuropharmaceuticals. Monoclonal antibodies (MAb) represent another type of proteins which can be used potentially as diagnostic or therapeutic agents. However, the poor permeability of peptides and proteins through biological membranes (*e.g.* cell membranes) is among the principal reasons, why these compounds have not yet gained the significance as neuroactive drugs in practice which theoretically could be expected from their bioactivity. The blood-brain barrier (BBB) consists of tightly sealed cell membranes and prevents the free access of large and/or hydrophilic substances from blood to brain. There are, however, active uptake systems for some endogenous peptides and proteins present at the BBB, which may be exploited in a physiological approach of drug delivery to the brain. After an introduction to the physiology of the BBB, an overview of potential strategies of drug delivery is given. The physiological approach is presented with examples, showing how peptides and proteins can be modified to achieve pharmacologically significant brain concentrations after systemic administration.

1. Introduction

1.1. *Peptides and Proteins as Potential Neuropharmaceuticals*

As a result of advanced analytical methods in biochemistry and the advent of receptor chemistry and molecular biology, the past two decades have seen an explosion in our knowledge about peptides and proteins as endogenous signal transmitters. Many dozens of hormones, neurotransmitters, and growth factors with peptide structure have been identified, and for most of them corresponding specific receptors are known. If a given peptide is either present within the central nervous system (CNS), or has actions on the CNS, it is categorized as a 'neuropeptide'. Although the physiological functions of many neuropeptides have not yet been elucidated in detail, it is clear that they exert highly specific and potent effects on neuronal systems. Synthetic agonists and antagonists and biotechnologically produced proteins may offer a wide array of neuropharmaceuticals for diagnosis and treatment of disorders of the brain (*Table*).

A well studied example is the family of endogenous opioids, *i.e.*, neuropeptides with opiate-like activity, which are widely distributed both within the CNS and the peripheral nervous system. There are at least three different classes of opioid receptors, designated as the μ-, δ-, and κ-receptor, each with subclasses. These systems are thought to be involved at various levels of the CNS in the processing of painful stimuli (analgesia) [1]. Since the discovery of the endogenous opioids (enkephalins, endorphins, dynorphins), a lot of research efforts have been made to develop peptide-based analogs as neuropharmaceutics with higher specificity and less side effects compared to classical opiate alkaloids such as morphine [2].

Another example is the field of growth factors, that includes the family of neurotrophins (*e.g.* nerve growth factor, NGF; brain derived neurotrophic factor, BDNF, and others). These factors play important roles in the development of the nervous system and in survival and regeneration of neurons. Therefore, great interest is currently focussed on the potential use of neurotrophic factors in neurodegenerative diseases, such as *Alzheimer*'s disease (AD) or *Parkinson*'s disease [3].

Finally, yet another class of proteins with great potential value as (neuro)-pharmaceuticals are the monoclonal antibodies [4]. These molecules can recognize and bind antigens with high specificity and affinity. Therefore, they can be used as diagnostic agents *in vitro* (*e.g.* immunoassays) and *in vivo* (*e.g.* after radiolabeling in imaging techniques in nuclear medicine). Moreover, the function of viral antigens or tumor antigens may be targeted with monoclonal antibodies in a therapeutic approach *in vivo*.

1.2. *Physiology of the Blood-Brain Barrier*

Before the promise of potent and specific neuropharmaceuticals derived from peptides and proteins can be realized, however, a drug delivery problem has to be solved [5]. The fact that none of the hundreds of opioid analogs developed so far has been introduced into clinical practice is mainly due to the pharmacokinetic problems encountered with peptide drugs in general: peptides and proteins range in molecular weight from several hundred

Table. *Potential Neuropharmaceuticals with Biooligomeric Structure*

Drug	Blood-brain barrier transport
peptides	No
recombinant proteins	No
monoclonal antibodies	No
antisense oligonucleotides	No
small molecules	
lipid-soluble, MW < 700 Da	Yes
lipid-insoluble or MW > 700 Da	Minimal

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daltons (*e.g.* enkephalins) to many kilodaltons (*e.g.* NGF with 26 kDa; MAb of the immunogobulin G (IgG) class with 150 kDa). In addition, they often have a high hydrogen bonding potential. Both size and hydrophilicity impede permeability through membranes (see below). In order to reach their target within the CNS, systemically administered drugs have to cross the blood-brain barrier (BBB) (*Fig. 1*) [6]. The physiological function of the BBB is to provide the CNS with a constant internal milieu. A second barrier, which separates the CNS compartment from the rest of the body, is the blood-cerebrospinal fluid barrier (B-CSF-B). Compared to the BBB, the surface area of the B-CSF-B is approximately 5000-fold smaller. This barrier is structurally and physiologically distinct from the BBB (*Fig. 1*), although the two barriers are often confused in the literature. Because the B-CSF-B is of minor importance for the delivery of peptides and proteins [5] to the brain tissue, it will not be further discussed here.

The anatomical substrate of the BBB is the wall of the blood vessels, in particular the brain capillaries, which supply blood to the brain tissue (*Fig. 1*) [7]. The inside of the blood vessels is lined by a single layer of endothelial cells, and the endothelial lining of the brain microvasculature is different from that in other organs. Brain capillary endothelial cells are connected to each other by complex strands of tight junctions, *i.e.* fusion zones between the cell membranes of neighboring cells. In that way, the lumen of the vessels is virtually covered by a continous sheet of plasma membrane, which is separated by the endothelial cell cytoplasm (on average only 300 nm in diameter) from a second continous sheet of cell membranes at the ablumenal side. Because the cell membrane consists of a lipid bilayer, the BBB may be portrayed in simplified terms as a double lipid bilayer. Unlike in other organs, the endothelial cells of the brain microvessels have no fenestrations or pores, and there is only negligible pinocytosis (uptake of small vesicles filled with fluid) [6]. Other cells, which are in close contact with endothelial cells on the brain side (= ablumenal), such as astroglial cells (the equivalent of connective tissue in the CNS), pericytes (presumably related to or derived from microglial cells or smooth muscle cells), and neurons, contribute to the properties of the BBB by inducing the specialization of the endothelial cells by mechanisms that are incompletely understood. Based on the structure, the BBB should only allow the free passage of lipophilic substances by the mechanism of diffusion, but not of hydrophilic substances [5][6][8]. In a first approximation this is actually the case. Classical neuroactive drugs, such as benzodiazepines, neuroleptics, and tricyclic antidepressive agents, are all small lipophilic molecules. In contrast, there are hydrophilic drugs, such as acetylsalicylic acid or benzylpenicillin, which show very poor permeability at the BBB under physiological conditions [9].

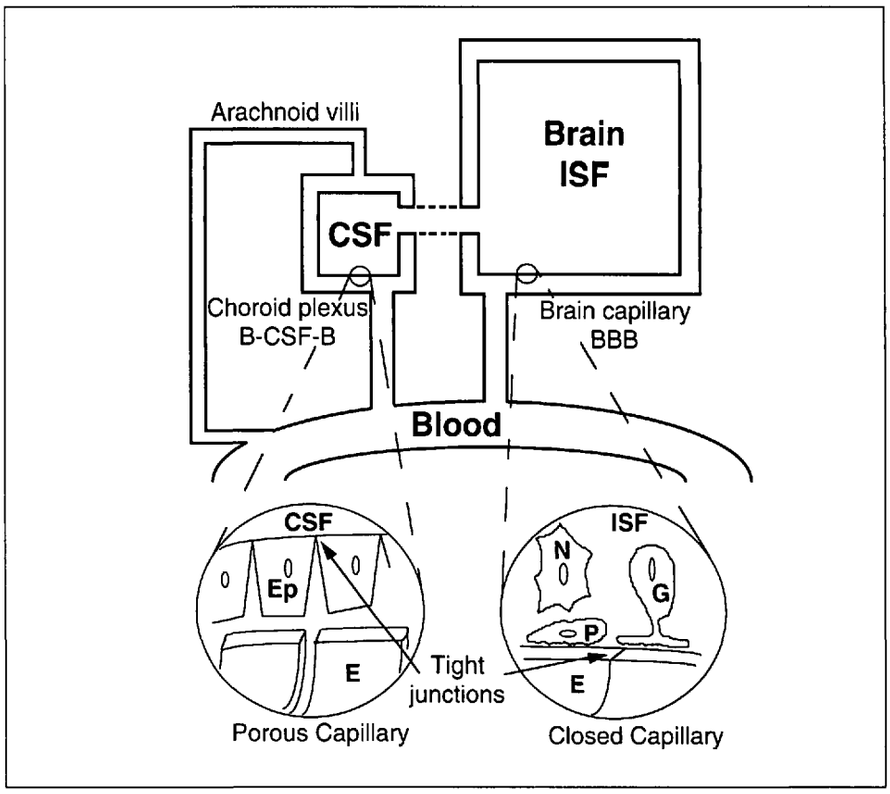

Fig. 1. *The two central extracellular compartments of brain,* i.e., *cerebrospinal fluid* (CSF) *and brain interstitial fluid* (ISF), *are segregated from blood by the choroid plexus or blood-CSF barrier* (B-CSF-B) *and by the brain capillary or blood-brain barrier* (BBB), *respectively.* Although the two extracellular fluid compartments are anatomically contiguous, there is a functional barrier preventing complete equilibration between these two fluid compartments. The presence of tight junctions on the apical or ventricular side of the ependymal cells (Ep) retards the further distribution of circulating substances into the CSF. It is estimated that the capillary surface area in the vast proper of brain perfused by capillaries with endothelial (E) tight junctions is *ca.* 5,000-fold greater than the surface area of the capillaries perfusing regions at the B-CSF-B. N, neuron; G, glia cell; P, pericyte.

Because many of the required nutrients of the brain (glucose, amino acids, nucleotides, and others) are hydrophilic and would cross the BBB by diffusion only poorly, the endothelial cells are endowed with membrane bound specific transport proteins for the facilitated uptake of these substrates from the blood (*e.g.* the glucose transporter called GLUT-1) [10]. These transporters bind their substrate molecule and temporarily open a pore, allowing the passage of the substrate across the cell membrane. For larger molecules such as peptides and proteins there is no uptake through pores. Instead, there are specific receptors for some peptides and proteins present at the lumenal side of the BBB, which promote cellular uptake by a mechanism called receptor-mediated endocytosis, *i.e.* the invagination and budding off of parts of the cell membrane to form small vesicles (endosomes) [11]. The transcellular passage of substance (transcytosis) is then completed by a corresponding mechanism, called exocytosis, at the ablumenal membrane. An example of a protein taken up at the BBB by this mechanism is transferrin, the transport protein for iron in the blood plasma [12].

2. Brain Drug Delivery Strategies

The potential approaches for the delivery of drugs to the brain can be classified into three categories: *i*) neurosurgical or invasive, *ii*) pharmacological, *iii*) physiological. A comprehensive description of all three strategies is beyond the scope of this essay. Instead, the first two categories will be briefly discussed here, and the following part will concentrate on the physiological approach.

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2.1. *Neurosurgical Delivery Strategies*

Invasive delivery strategies include methods that temporarily open the BBB from the blood side in a non-specific manner. The short term infusion of hyperosmolar solutions (*e.g.* 2M mannitol) directly into the arteries supplying the brain (internal carotid artery or vertebrate artery) disrupts the BBB by osmotic shrinking of the endothelial cells and allows the passage of small molecules (*e.g.* cytostatic drugs) and large molecules (*e.g.* antibodies) into brain tissue for minutes to hours [13]. The procedure is performed under general anesthesia and it requires the catheterization of the carotid or vertebral artery. By the same route the BBB may be transiently opened by the intraarterial administration of vasoactive substances such as leukotrienes [14].

The other invasive strategy employs the direct instillation of drugs into the cerebrospinal fluid or into the brain tissue itself. These techniques require the neurosurgical placement of catheters into the cerebral ventricles or into the brain. Between the cerebrospinal fluid and brain there is no tight cellular barrier. However, the distribution into deeper tissue layers is impeded by several mechanisms: *a*) There is a relatively rapid turnover of the CSF, which is secreted by a specialized tissue inside the ventricles (choroid plexus) and reabsorbed at the surface of the brain into the venous blood. In humans, the whole volume of CSF (*ca.* 140 ml) is exchanged in 4–5 h [5][6]. *b*) Tissue concentrations achieved by diffusion fall off with the square root of the diffusional distance, and the coefficient of diffusion decreases with increasing molecular size. Therefore, it takes a molecule such as glucose (180 Da) *ca.* 12 h to cover a distance of 5 mm, but for a protein such as myoglobin (17.5 kDa) it takes 2.7 days [15]. *c*) Along the diffusional path, there may be cellular uptake and/or metabolism. As a result of the points outlined above, there is an exponential decline in the tissue concentrations of even small molecular weight drugs after intraventricular application with increasing distance from the surface of the brain [16]. Therefore, efficient drug delivery can only be achieved when the target is close to the surface. Analogous is the situation after local administration directly into the brain tissue, where drug distribution by diffusion to surrounding tissue is limited.

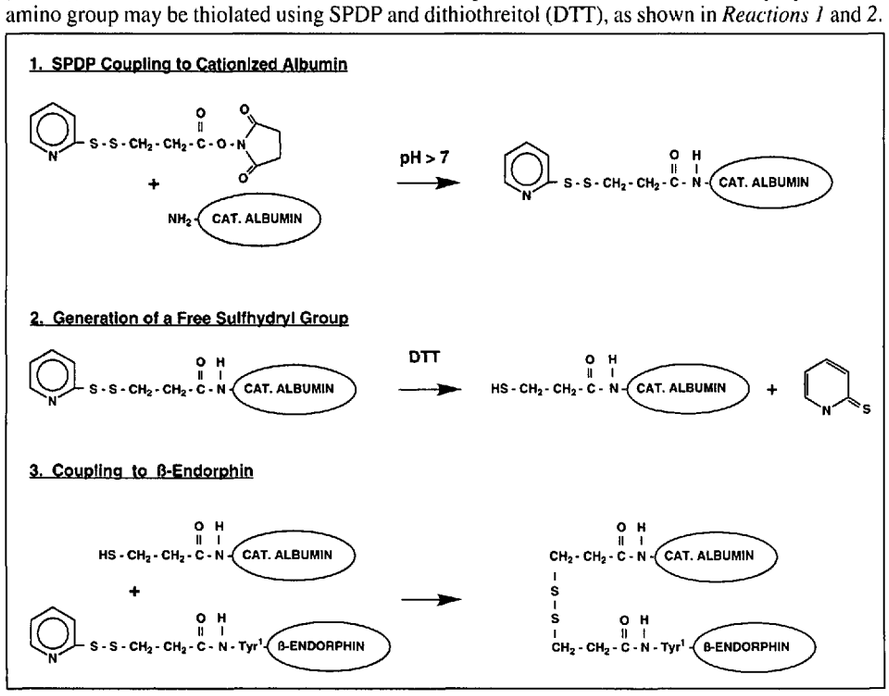
2.2. *Pharmacological Delivery Strategies*

In order to facilitate the uptake of small peptides through the BBB by diffusion, chemical modifications may be introduced which increase the lipophilicity. There are several methods to measure the lipophilicity of a compound. Frequently used parameters are partition coefficients between a lipid and an aqueous phase, such as the octanol/water partition coefficient (also expressed as log P) [17], or hydrogen bond numbers [18]. The latter can be experimentally derived from partition coefficients or be estimated from the functional groups present in the molecule. The impact of lipophilicity on BBB permability of small molecules may be demonstrated with the series of opiate alkaloids: compared to morphine, codeine (6-methylmorphine), and heroin (3,6-diacetylmorphine) have a decreased hydrogen bonding potential and thereby increased lipophilicity. Correspondingly, codeine and heroin cross the BBB approximately tenfold and one hundred-fold better than morphine [19]. In the peptide field, there are mainly two approaches to lipidization. One is the derivatization of functional groups, *e.g.* alkylation of OH, NH_2, or COOH, or the cyclization of small peptides. The alternative is the formation of prodrugs by conjugation at the amino or carboxy terminus of lipophilic moieties such as adamantane [20], cholesterol, or dihydrotrigonellinate [21]. In the case of dihydrotrigonellinate, a second consequence of the derivatization is the possibility of sequential metabolism, *i.e.*, the peptide drug is trapped inside the target compartment (brain) after crossing the BBB as a prodrug by tissue-specific metabolism of the conjugate [21].

High lipophilicity is, however, not in every case a guarantee for good BBB permeability. Some substances, which would be expected to pass through the BBB by diffusion owing to high lipid solubility, such as the immunosuppressant cyclosporin, actually show only marginal brain uptake [22]. Cyclosporin is a cyclic natural undecapeptide (molecular weight 1203 Da) which is extremely well lipid soluble due to some unusual structural features. The failure to show significant BBB permeability may be due to either an upper molecular-weight limit in diffusion-mediated permeability of *ca.* 500–700 Da [23], or to the presence of an active pump mechanism at the BBB in the outward direction. It is known, that cyclosporin and a number of other substances with poor apparent BBB permeability are substrates of the *P*-glycoprotein, the product of the multidrug resistance (mdr) gene. *P*-glycoprotein is a pump in the cell membrane, and its presence and function at the BBB has recently been postulated [24].

A quantitative framework of what can be expected in terms of brain delivery by chemical design strategies of small peptide molecules, such as lipidization, may be derived from the measurement of brain uptake of a metabolically stable opioid peptide, DALDA (molecular weight 616) [25a]. DALDA is relatively well lipid-soluble (log P value of –1.5 compared to –3.3 for sucrose), and it crosses the BBB

Scheme 1. *A Primary Amino Group on Cationized Albumin May Be Coupled to a Sulfhydryl Group on β-Endorphin Using the Disulfide Crosslinker Reagent* N-*Succinimidyl 3-(2-pyridyldithio)propionate (SPDP), as Shown in* Reactions 1 *and* 3. If one of the proteins lacks a free surface sulfhydryl, then an amino group may be thiolated using SPDP and dithiothreitol (DTT), as shown in *Reactions 1* and 2.



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by diffusion to yield a brain concentration of 0.02% of the injected dose per g brain (%I.D./g), an extent which permits a threshold pharmacological effect (central analgesia) after the peripheral administration of an intravenous dose of 5 mg/kg to rats. For comparison, the peak brain concentration under equivalent conditions of the classical neuroactive drug morphine is *ca.* 0.06% I.D./g [19].

Pharmacokinetic considerations let it appear unlikely, that a substantial increase of brain delivery (or a substantial reduction in the required systemic dose) can be achieved by the lipidization strategy. The pharmacokinetic principles of prime importance for uptake through the BBB were recently reviewed [25b,c]. Brain uptake (%I.D./g) of a compound is a function of both, the BBB permeability, and the area under the plasma concentration-time curve (AUC). Permeability may be expressed as the permeability surface area (PS) product (units ml/min/g). The corresponding units of AUC are %I.D. · min/ml. It can be seen from the equation:

$$\%I.D./g = PS \cdot AUC$$

that the effect of an increase in BBB PS, *e.g.* by synthesis of an analogue with higher lipophilicity, may be offset by a concomitant decrease in AUC (equivalent to an increase in the systemic clearance of the substance). The latter may be due, *e.g.* to increased uptake into peripheral tissues, secondary to overall increased permeability through cell membranes.

2.3. *Physiological Delivery Strategies*

This approach exploits uptake mechanisms present at the BBB for the transport of nutrients and hormones (see *Sect. 1.2*). A neuroactive drug in widespread clinical use that falls into this category is the α-amino acid, L-dopa, used in the therapy of *Parkinson*'s disease [26]. In this neurodegenerative disease, specific brain regions lack the neurotransmitter dopamine. A substitution therapy with dopamine is impossible, because it is poorly transported through the BBB. The precursor of dopamine, L-dopa, however, has affinity to the BBB transporter for large neutral amino acids. After uptake, L-dopa is then enzymatically converted to dopamine by aromatic amino acid decarboxylase, which is present in brain. BBB nutrient carriers such as the L-amino-acid transport system play a role in the brain uptake of small molecule drugs with structural similarity to the natural ligands ('pseudoneutrients', discussed in [25b]). The size of a peptide or protein would not allow passage through the transmembrane pores or tunnels controlled by the nutrient carriers. However, several receptors at the BBB-mediating uptake of peptide and protein hormones have recently been described, such as the insulin receptor, the receptors for insulin-like growth factors I and II, the transferrin receptor, and the receptor for interleukin-1 [27]. The receptors typically show a high degree of specificity for the respective ligand. These transport systems may be exploited to piggy-back drugs across the BBB by a mechanism called receptor-mediated transcytosis [5][11]. A promising approach to drug delivery by this mechanism is the synthesis of chimeric peptides, where a non-transportable peptide drug is bound to a vector, which has affinity to a receptor at the lumenal side of the BBB and mediates brain uptake (*Fig. 2*). With currently used vectors (see below) the amount of drug which can be delivered to brain is at least one order of magnitude higher than uptake by diffusion of small peptides such as DALDA (*i.e.* brain concentrations > 0.2 % I.D./g are achieved). For larger proteins which cannot at all penetrate the BBB spontaneously, the ratio is even better.

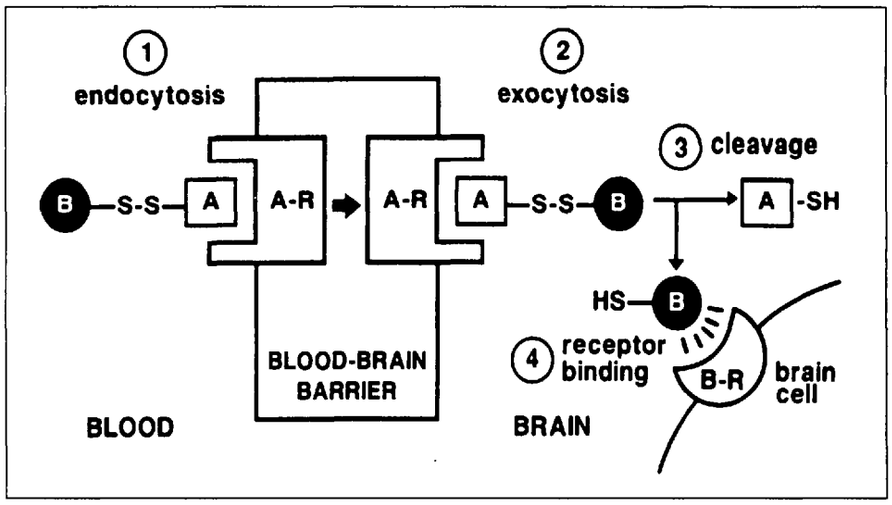

Fig. 2. *The delivery of chimeric peptides through the BBB is viewed as a process composed of 4 steps: 1) Receptor- or absorptive-mediated endocytosis of the blood-borne chimeric peptide into brain endothelial cytoplasm; 2) exocytosis of the chimeric peptide into the brain interstitial fluid; 3) cleavage of the disulfide bond liberating unconjugated transport vector and pharmacologically active peptide; 4) binding of the pharmacologically active peptide with its receptor on brain cells.* Abbreviations: A, transport vector, *e.g.* cationized albumin; B, non-transportable (pharmacologically active) peptide, *e.g.* β-endorphin; A-R, receptor for the vector on endothelial cells; B-R, peptide B receptor (from [32]).

Another mechanism, which is not as specific as receptor-mediated uptake, is the so-called absorptive mediated transcytosis. It applies to lectins (glycoproteins of plant origin) [28] and to certain cationic proteins (*i.e.* proteins with an alkaline isoelectric point (p*I*)) [29]. Cationization causes uptake through cell membranes by triggering electrostatic interactions of positive charges on the protein surface with negative charges displayed on the cell membrane (in the case of the BBB on the lumenal cell membrane of endothelial cells) [30]. The further steps of transcellular transport of cationized proteins are analogous to receptor-mediated transcytosis. Therefore, cationized proteins may be used either as vectors for peptide drugs (see *Sect. 3.1*) or as drugs in their own right (see *Sect. 3.3*).

3. The Chemical Synthesis of Vectors and Chimeric Peptides

3.1. *Direct Linker Strategy for Chimeric Peptides*

A number of chemical approaches to crosslinking of two peptides or proteins has been developed. The first chimeric peptide specifically designed for brain uptake was a conjugate of cationized albumin (vector) and the opioid peptide β-endorphin (nontransportable drug) [31] [32]. In this case, a disulfide linkage was introduced. *Scheme 1* shows the steps involved. Both albumin and β-endorphin were first derivatized with the heterobifunctional crosslinker *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) [33]. If the peptide drug to be coupled already had a free thiol group, it could be directly coupled to the SPDP-activated vector. That was not the case with β-endorphin. Therefore, a free thiol group on the albumin was generated by treatment with dithiothreitol (DTT). The thiolated albumin was then reacted with SPDP-activated β-endorphin.

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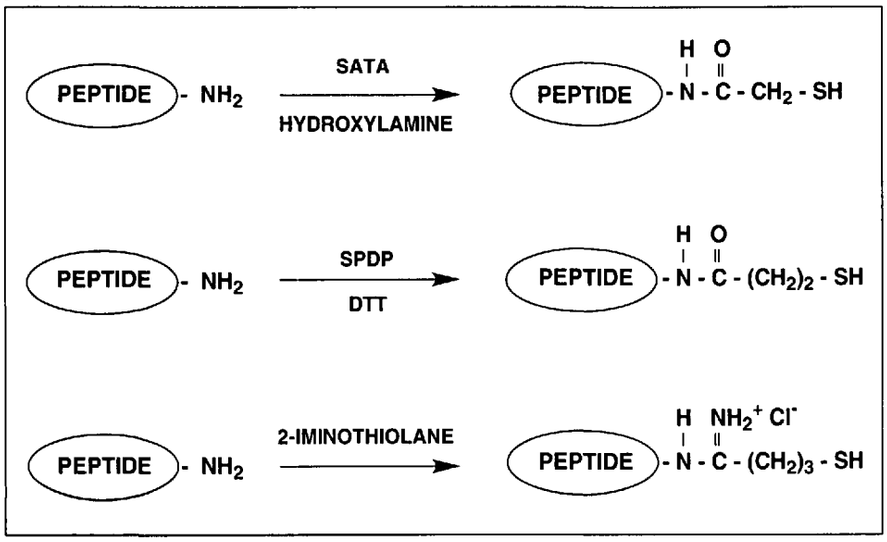
Scheme 2. *Alternative Methods for the Introduction of Thiol Groups at Primary Peptide Amino Groups.* SATA = *N*-succinimidyl 2-(acetylthio)acetate; SPDP = *N*-succinimidyl 3-(2-pyridyldithio)-propionate; DTT = dithiothreitol. Modified from [5b].



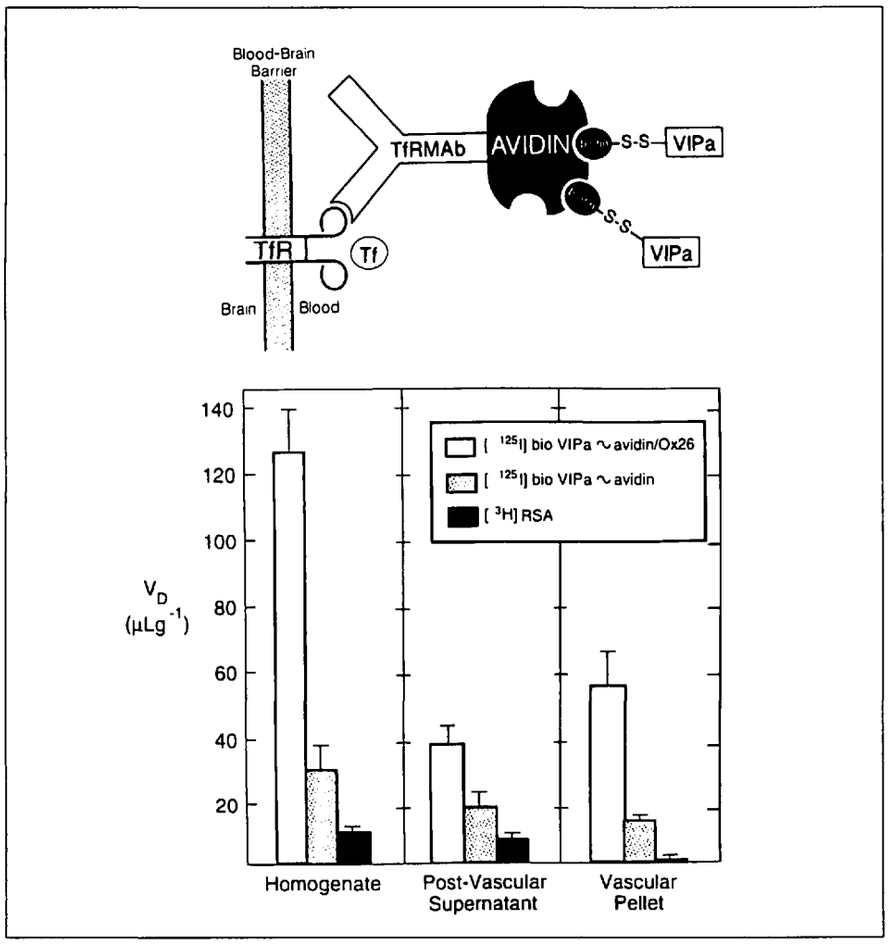


Fig. 3. *Scheme of a covalent conjugate of a monoclonal antibody* (TfRMAb) *to the transferrin receptor* (TfR) *and avidin as a brain drug transport vector* (upper). (The avidin moiety binds the biotinylated peptide drug, in this example, an analogue of vasoactive intestinal peptide, VIPa. Tf, transferrin, does not compete with the binding site of the monoclonal antibody.) *Brain uptake in rats of tracers* (see Inset) *as determined by an internal carotid artery perfusion/capillary depletion technique* (lower). (Tissue uptake is expressed as volume of distribution in µl/g at the end of a 10 min perfusion with tracer solution containing either ^{125}I-bioVIPa-avidin-OX26 and ^{3}H-labeled rat serum albumin ([^{3}H]RSA) or ^{125}I-bioVIPa-avidin and [^{3}H]RSA (n = 3 per group). Results are shown for whole tissue homogenate, postvascular supernatant of homogenate, and vascular pellet. The results of the two groups for [^{3}H]RSA, which is a nonpermeable marker for brain intravascular volume, were not significantly different and were combined for the evaluation.) Bars represent means ± SE. From [47].

The rationale for using disulfide linkers is as follows: After systemic administration (*e.g.* intravenous injection) of a chimeric peptide drug, the conjugate is first in the blood compartment. A linkage with either spontaneous lability in plasma or susceptibility to cleavage by plasma enzymes would result in the premature release of the peptide moiety from the vector. Disulfide bridges are relatively stable in plasma but labile inside cells [34]. As shown in *Fig. 2*, the peptide drug should only be released after passing through the BBB. With the β-endorphin-albumin conjugate it could be demonstrated, that there are enzymes present in brain tissue which are able to cleave the chimeric peptide rapidly [32]. On the other hand, isolated brain capillaries (*i.e.* an *in vitro* model of the BBB) did not cleave the disulfide bridge. Recently the cleavage of a disulfide-linked chimeric peptide could also be confirmed *in vivo* [35] (see *Sect. 3.2*).

As shown above, the lack of free -SH groups in peptides or proteins requires the thiolation. The approach with SPDP and DTT treatment requires the efficient removal of the reducing agent prior to the coupling step, otherwise the formation of the disulfide linkage will be compromised. Alternative methods for the thiolation of peptides at amino groups (α-amino group at the NH_2 terminus or ε-amino group in lysine residues) with reagents that are readily commercially available are shown in *Scheme 2*. It should be noted that all derivatives leave a molecular adduct on the peptide molecule even after cleavage of the disulfide bridge. Therefore, the impact of such an adduct on the bioactivity of the peptide drug has to be checked. The number of sulfhydryl groups introduced into the molecule may be experimentally determined by quantitative assays (*e.g.* with 5,5'-dithiobis(2-nitrobenzoic acid) = DTNB, *Ellman*'s reagent) [36].

It is also possible to thiolate carboxy groups (at the COOH terminus or on internal aspartate or glutamate residues), if derivatization of amino groups in a given protein is not feasible. In the case of NGF, the coupling through amino groups lead to loss of bioactivity [37]. Consequently, the synthesis of a chimeric peptide for brain delivery of NGF involved a coupling strategy through carboxy groups (*Scheme 3*) [38]. The OX26 antibody was used as a vector [39]. OX26 is a mouse MAb to the rat transferrin receptor. The receptor is highly abundant on brain capillaries [27d]. Because of its high binding affinity for transferrin (K_D = 6 nM) and the comparatively high plasma concentrations of en-

dogenous transferrin (25 μM), the receptor is almost saturated under physiological conditions [40]. Therefore, transferrin itself may not be a useful vector. In contrast, OX26 binds to an epitope distinct from the transferrin binding site, *i.e.* the binding of OX26 is not competed by transferrin.

Using OX26 as a vector and a suitable animal model for the *in vivo* demonstration of pharmacological effects, the vector mediated brain delivery of NGF has been shown [37]. Due to the low K_D value (10 pM) for the binding of NGF to its high-affinity receptor, a small dose of 6.2 μg (2.3 nmol) NGF per injection was sufficient to achieve the desired biological effect (the survival of cholinergic neurons in a certain CNS-graft model in rats). The bioactivity of the NGF-OX26 chimeric peptide could recently be confirmed in another *in vivo* model [41].

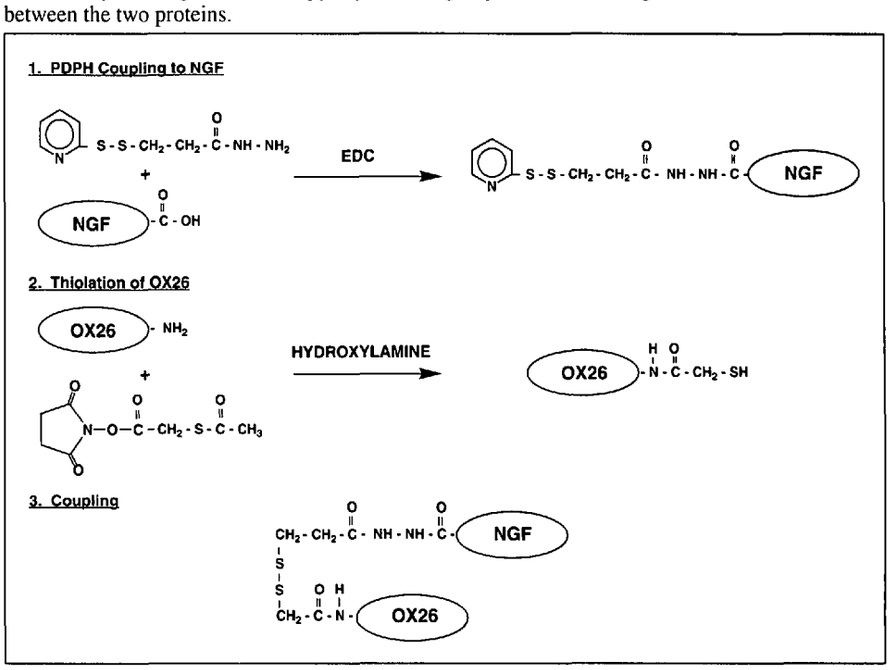
3.2. *Avidin-Biotin Linker Strategy*

Direct crosslinking of peptide and vector has the disadvantage that the linker chemistry needs to be optimized in each individual case and the overall yield may be low (in the range of 10–15% [31]) due to numerous purification steps involved. Progress in this respect could be achieved by the introduction of universal drug delivery vectors based on the avidin-biotin technology [42]. The interactions of biotin (Vitamin H) with the avian protein avidin present in egg white or with its bacterial analogue, streptavidin, represent the strongest noncovalent binding of ligand to a protein with an affinity constant of 10^{15} M^{-1} and a half life of dissociation of the complex of 89 days. Moreover, the avidin biotin complex is stable over a wide range of physical and chemical conditions (*e.g.* pH 2–13). Because of these characteristics avidin-biotin systems are widely used in the biochemical field. A variety of reagents is readily available for the efficient biotinylation of peptides and non-peptides (*e.g.* nucleotides) at different functional groups (*e.g.* amino groups, hydroxy group or carboxy groups).

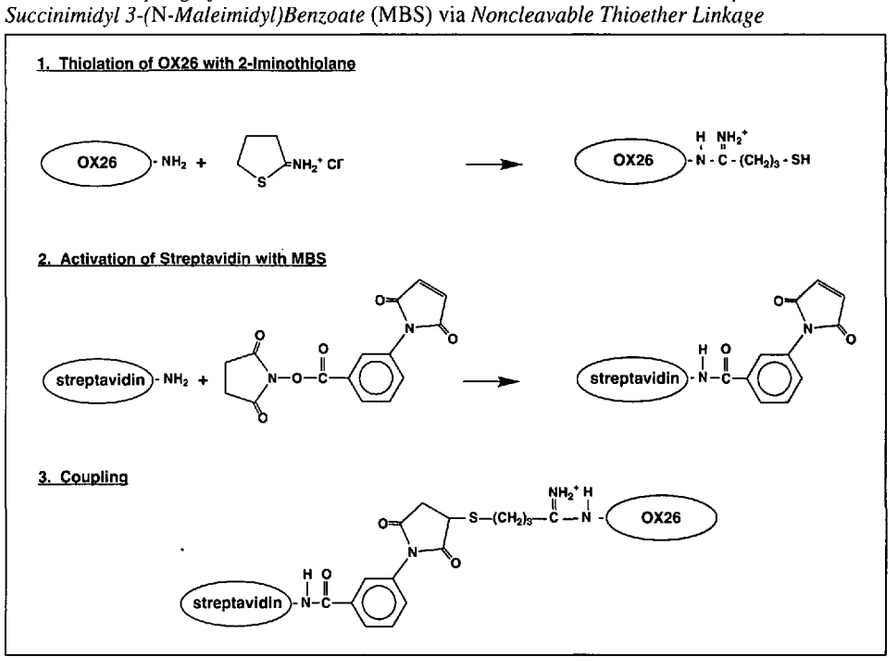
A vector construct obtained by chemical conjugation of the OX26 antibody and avidin or streptavidin (OX26-AV, OX26-SA) is shown in *Fig. 3* and *Scheme 4*. *Fig. 3* also gives an example of the brain delivery of a biotinylated peptide ligand using the vector. The linkage between the antibody and avidin or streptavidin is accomplished by a noncleavable thioether bond (*Scheme 4*). Initially, avidin was used, which is a basic protein with a p*I* of *ca.* 10 [42]. Due to this cationic nature it can actually promote cellular uptake of biotinylated ligands *in vitro* by absorptive-mediated endocytosis [43]. It is, however, rapidly removed from the circulation after *in vivo* administration by uptake predominantly in liver and kidney, and is, therefore, not useful as a brain delivery vector [44]. By comparison of the brain delivery of native OX26 with OX26-AV, it was noticed that the cationic nature of avidin also had a negative effect on the pharmacokinetics of the conjugate, decreasing plasma AUC and brain uptake [45]. The substitution of avidin with a chemically neutralized form (neutral avidin) or with streptavidin (which has a natural p*I* of 5–6) improved the brain delivery of the vector to the level of native OX26 [46].

The OX26-AV vector system can potentially bind any biotinylated drug. How-

Scheme 3. *Pyridyldithiopropionohydrazide* (PDPH) *was Used to Attach a Thiolreactive Group to NGF by Means of Carboxy Groups that Had Been Activated with 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide* (EDC). SATA (*N*-succinimidyl 2-(acetylthio)acetate) was reacted with lysine ε-amines on the antibody to introduce a protected sulfhydryl group. The sulfhydryl group on the antibody exchanged with the 2-pyridyl-sulfide group on NGF, forming a cleavable disulfide bond between the two proteins.



Scheme 4. *Coupling of Thiolated OX26 with 2-Iminothiolane to Activated Streptavidin with* N-*Succinimidyl 3-(*N*-Maleimidyl)Benzoate* (MBS) via *Noncleavable Thioether Linkage*



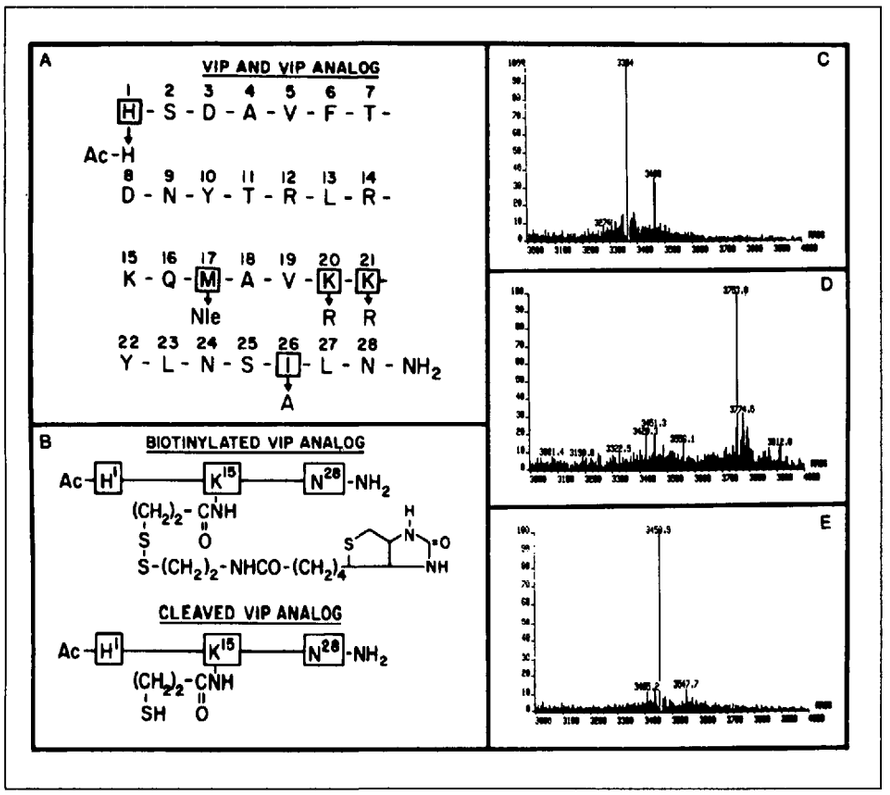


Fig. 4. *Amino-acid sequence of native VIP and modifications in VIPa as indicated by arrows* (A); *Structure of bioVIPa and desbioVIPa* (B); *molecular mass as determined by FAB-MS of HPLC purified VIPa* (C), *bioVIPa* (D), *and desbioVIPa* (E). From [47].

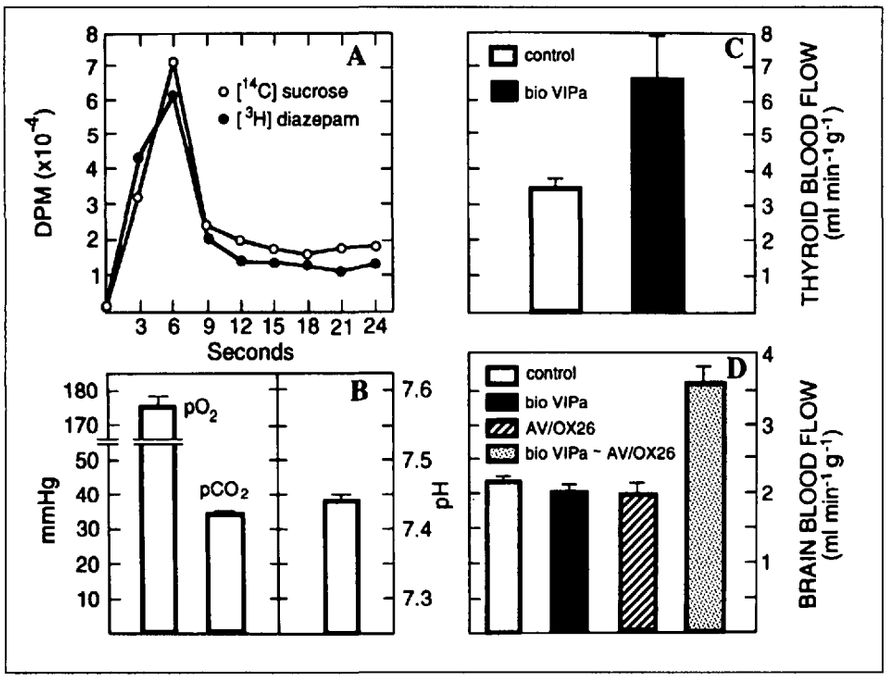


Fig. 5. *Measurement of tissue blood flow after 10 min intracarotid infusions of vehicle* (control), *bioVIPa* (12 µg/kg), *avidin-OX26* (430 µg/kg), *or bioVIPa-avidin-OX26* (12 µg of bioVIPa and 430 µg of avidin-OX26 per kg) *in N_2O-anesthetized rats.* A) Arterial blood radioactivity of [^{3}H]diazepam or [^{14}C]sucrose was determined in sampling periods of 3 sec after i.v. bolus injection. B) No significant differences in arterial blood gas measurements of the four treatment groups listed in D were observed and means ± SE for all animals (n = 16) are shown. C) Rate of thyroid blood flow is increased by systemic administration of bioVIPa over 10 min infusion period. D) Brain-blood flow is increased 65% ($P < 0.0025$; Student's test versus control) by systemic administration of bioVIPa coupled to the avidin-OX26 (AV/OX26) vector, whereas administration of either bioVIPa or AV/OX26 vector alone causes no change in brain blood flow relative to control. No difference in blood flow between right and left hemispheres was found. Data are means ± SE (n = 4 rats per group; body weight, 270–280g).

ever, some criteria have to be met in the design of a biotinylated ligand suitable for *in vivo* drug delivery. An example of a neuropeptide (an analogue of the Vasoactive Intestinal Peptide, VIP) specifically designed for this type of vector is shown in *Fig. 4* [47]. The rationale for using a VIP analogue in these experiments is explained below.

As depicted in *Fig. 3*, avidin (and streptavidin) has four independent binding sites for biotin. Monobiotinylation of the ligand is, therefore, required to avoid the formation of high molecular weight aggregates with the vector, which would be rapidly cleared from the blood *in vivo*. In the VIP analogue all but one possible conjugation sites for the biotinylating reagent NHS-SS-biotin (=sulfosuccinimidyl 2-(biotinamido)ethyl-1,3'-dithiopropionate) were eliminated by amino-acid substitutions or blocking (acetylation of the N-terminal α-amino group). A temporary protection during the biotinylation step of functional groups, which are important for bioactivity, may also be achieved by reversible blocking groups (*e.g.* Fmoc for N-terminal α-amino groups in opioid peptides [35]).

The finding cited above [37], that conjugation should not interfere with bioactivity, actually applied to a biotinylation. Therefore, based on known structure-activity relations of VIP, the biotinylation site was chosen in a region of minor importance for receptor binding and bioactivity (lysine at position 15). Some amino acids were substituted to increase the metabolic stability of the peptide. Using a suitable radioreceptor assay, the bioactivity of the VIP analogue and its biotinylated derivative could be demonstrated [47].

The ultimate test for the utility of any drug delivery approach is the demonstration of a pharmacologic effect. VIP is known to be involved in the regulation of cerebral blood flow (CBF) by vasodilation, and CBF is among the parameters of CNS function which are relatively easy accessible for quantitative measurement *in vivo*. It had previously been shown that the VIP receptors responsible for the vasodilation are localized on the brain side of the BBB. When the chimeric peptide consisting of the biotinylated VIP analogue coupled to OX26-AV was systemically infused in rats, a significant increase of CBF could be measured compared to controls, which received the same low dose of peptide (12 µg/kg body weight) without the vector (*Fig. 5*) [47].

NHS-SS-biotin introduces a disulfide bond (see *Figs. 3* and *4*). Therefore, the resulting chimeric peptide is cleavable. It

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could recently be shown with a biotinylated opioid analogue, Lys^7-dermorphin [35], which was selected to have the same structural features for coupling to OX26-AV discussed for the VIP analogue, that rapid *in vivo* cleavage from the vector in brain tissue takes place (*Fig. 6*), while the chimeric peptide was stable in plasma. At least in this case, the cleavage was actually necessary to restore the biological activity of the peptide. The activity was retained after biotinylation, but was significantly reduced by subsequent binding of the biotinylated peptide to avidin. An alternative to cleavable biotin linkers may be the use of linkers with even longer spacer arms than currently available (> 14-atom spacer) to avoid interference of avidin binding to the biotin moiety with the bioactivity of the peptide drug. Then the peptide could bind to its receptor while still being attached to the vector.

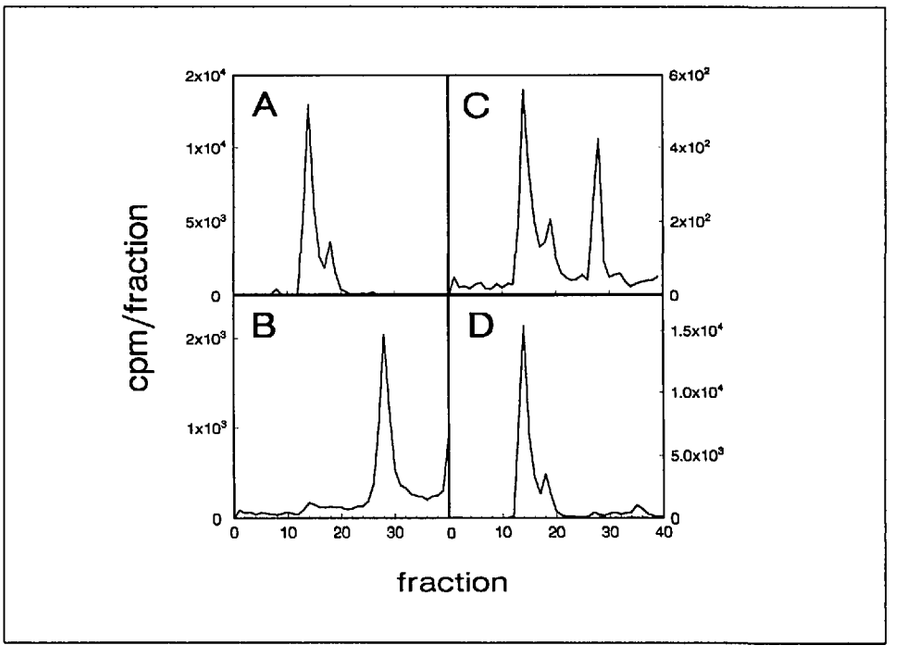
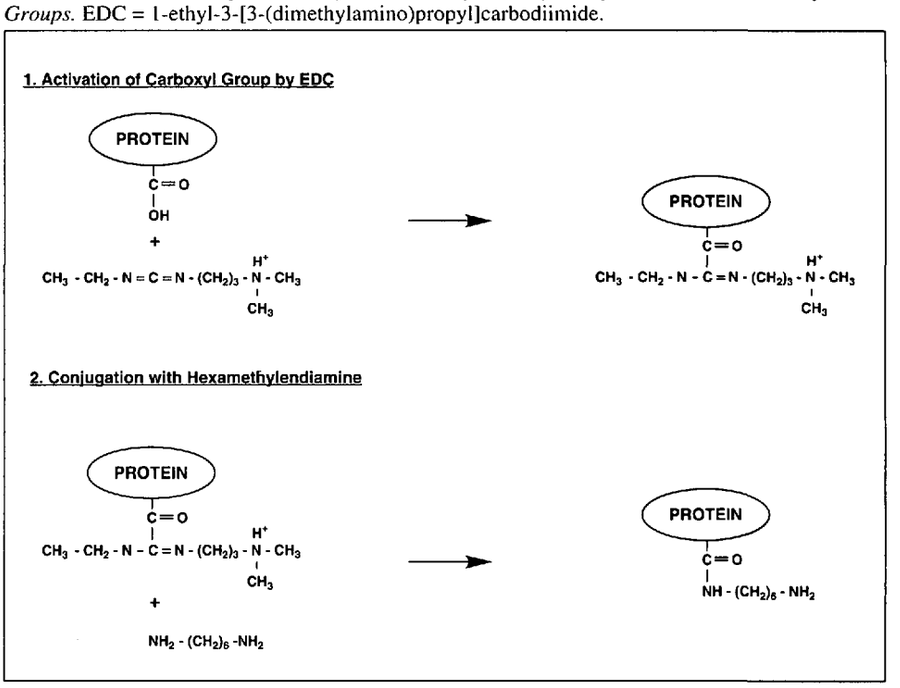


Fig. 6. *Gel filtration HPLC of ^{125}I-bio-SS-K7DA/NLA-OX26* (A), *and same tracer after treatment with 20 mM DTT* (B). (Cleavage of the S-S bridge leads to a shift of the activity from high molecular weight (vector bound) to low molecular weight (free peptide).) *Chromatogram of supernatant of brain homogenate following a 10 min brain perfusion with ^{125}I-bio-SS-K7DA/NLA-OX26* (C); *the control, where the tracer was added to fresh brain tissue in ice cold homogenization buffer before homogenization* (D). Fractions = 0.5 ml.

3.3. *Cationization of Proteins*

The chemical conversion of surface carboxy groups in a protein to extended primary amino groups can be accomplished as shown in *Scheme 5* [29][31][48a]. Isoelectric focussing is then used to measure the shift in the p*I* between the native and cationized protein. There are several variables which can be modified to achieve the desired degree of cationization, such as pH and the molar excess of the carbodiimide and hexamethylenediamine relative to the available COOH groups [31][48b]. The more acidic the p*I* of the native protein (*i.e.*, the higher the content of aspartate and glutamate residues in the sequence), the more readily can it be cationized. Under optimum conditions the cationization reaction should not crosslink protein molecules. The molecular weight of the modified protein may be checked by methods such as sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). When an antibody is cationized, the retention of binding affinity has to be checked, since COOH groups in the binding region could be derivatized. If the antigen is readily available (*e.g.* as a synthetic peptide) a site-protection protocol may be used where the antigen binding site of the antibody is protected during the cationization by the presence of bound antigen [48b].

Scheme 5. *Cationization of a Protein by Conversion of Carboxy Groups into Extended Primary Amino Groups.* EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

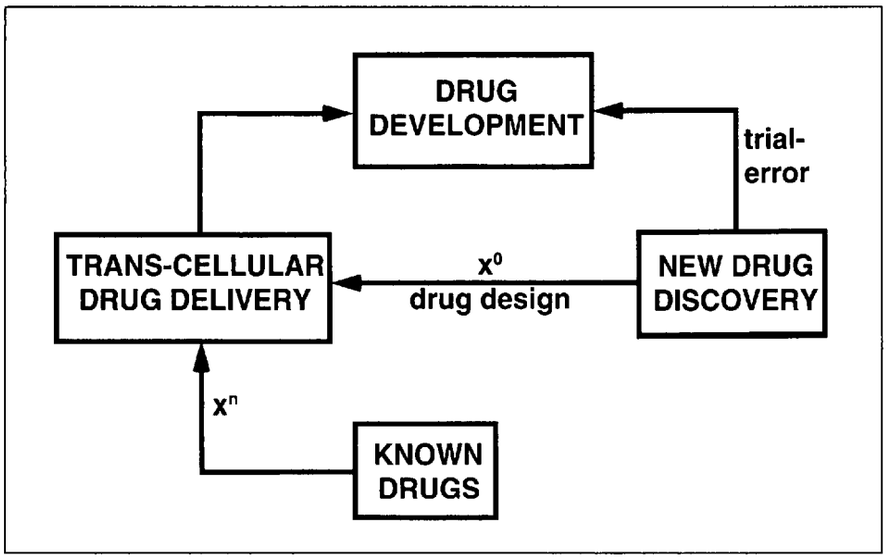


The feasibility of cationization to achieve brain delivery of proteins has been demonstrated with albumin and immunoglobulins from different species (bovine, mouse, rat, human). Natural polycationic proteins, such as avidin (see above) or histone can also show some degree of absorptive mediated brain uptake. It is remarkable, however, that a basic p*I* alone is not sufficient to induce brain uptake. A native MAb with a p*I* of 8.8 did not show any brain uptake, while cationization of this MAb to a p*I* > 9.5 caused significant uptake [48e]. Apparently, the cationic groups have to be present in a specific conformation to induce cellular uptake [5a].

The usefulness of cationized albumins as vectors (*Sect. 2.1*) can be improved by the avidin-biotin linker technology [49], and cationized polyclonal and monoclonal antibodies can be developed as diagnostic or therapeutic agents. Recently, a monoclonal antibody to β-amyloid, a marker in the brain of patients with *Alzheimer*'s disease, was cationized and radiolabeled. The

cationized MAb retained its high affinity for β-amyloid and was characterized *in vitro* and *in vivo* in animals [48b,c]. This could eventually facilitate early diagnosis of the disease by a noninvasive diagnostic imaging technique. Other antibodies directed against a viral regulatory protein (rev) of the Human Immunodeficiency Virus [50] and against a tumour antigen (ras) [48e] were shown to retain binding affinity after cationization. They displayed cellular uptake *in vitro* and brain uptake in animal models *in vivo*. If the biological activity which was already demonstrated *in vitro* in cell-culture models can be confirmed in suitable *in vivo* models, these studies may pave the way to future use of cationized antibodies for the therapy of viral infections and tumours of the CNS [48d].

Scheme 6. *The Potential Impact of Drug Delivery Strategies on Drug Development.* The trial-and-error approach typical for new drug discovery in the past usually resulted in smaller molecular weight, lipid-soluble drugs that penetrate cell membranes well. In contrast, rational drug-design strategies based on information on drug receptor interaction, will typically result in the discovery of hydrophilic molecules. The transcellular delivery of such new drugs must then be enhanced, or they will not succeed on the pathway from discovery of an active compound to actual drug development. Known drugs are often precluded from certain indications, *e.g.* treatment of CNS disorders, by poor transcellular delivery properties. The scheme emphasizes the benefit of developing effective transcellular drug delivery systems: single drugs (x^0) are generated by drug development from drug discovery programs, whereas multiple drugs (x^n) can result from a single drug delivery program. From [5a].



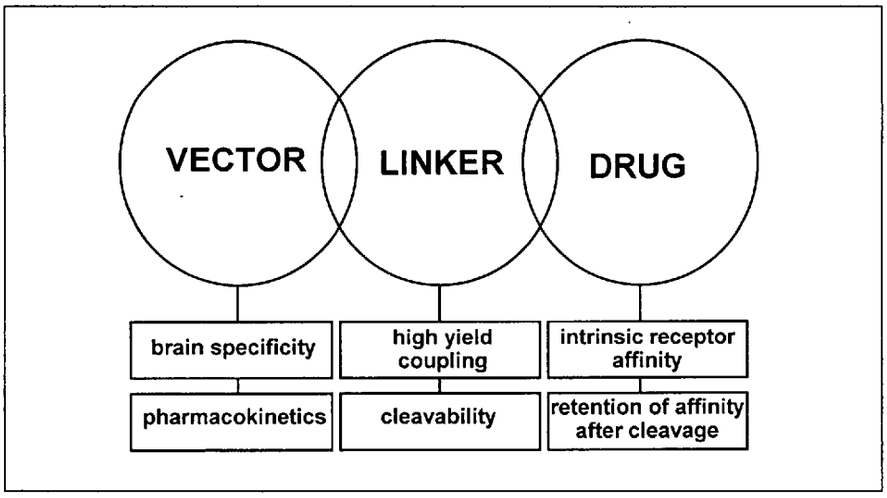


Fig. 7. *The three principal intersecting spheres of the chimeric peptide strategy include vectors, linkers, and drugs.* Crucial design considerations in the overall development of effective chimeric peptides include vector specificity for the brain, vector pharmacokinetics, high yield coupling and the issue of cleavability (linker strategies), and intrinsic receptor affinity of the drug following release from the transport vector. From [15].

4. Perspective

The importance of adequate brain drug delivery strategies in the overall drug development process, in particular in the development of neuropharmaceuticals, can hardly be overestimated. Until recently, there was rather an indifference and ignorance to the pharmacokinetic problem prevalent in the field. This is gradually changing as it becomes obvious that the potential drug products made available by modern biotechnology (*e.g.* recombinant growth factors, MAb, oligonucleotides) all show delivery problems *in vivo*. *Scheme 6* depicts the situation and emphasizes the equal importance of transcellular delivery and new drug discovery. The high cost of development and the time it takes to bring a new drug to the market, will prevent the chance to improve the delivery of an approved drug at a later stage, even if the pharmacokinetics of the original formulation are poor. The reason is, that the drug combined with a delivery system such as described in this review will have to pass through all stages of preclinical and clinical testing once again. Therefore, the delivery problem has to be studied and solved at the initial stages of new drug development. *Fig. 7* illustrates the complexity of the task using the development of optimized chimeric peptides for brain drug delivery as an example. On the vector side, the goals for the future are to improve the brain specificity and pharmacokinetics. The vectors investigated so far are not specific for brain relative to peripheral tissues. It should, however, be kept in mind in this context, that classical 'small molecule' neuropharmaceuticals, such as morphine, can by no means be regarded as brain-selective from a pharmacokinetic point of view (see *Sect. 2.2*). An evolution in vector discovery in terms of the efficiency of targeting brain can clearly be demonstrated: the recently described vector, MAb83-14, which is a murine monoclonal antibody to the human insulin receptor, yields a delivery of almost 4% I.D. per total brain (measured in the rhesus monkey [51]). Once receptors can be characterized at the BBB which are not present in the periphery, brain-specific targets for vector development will be available. Improvements in plasma pharmacokinetics can be achieved by rational vector design (*Sect. 3.2* [45][46]).

Genetic engineering may be applied to improve vectors and linker strategies. For example, the 'humanization' of antibodies will greatly reduce the potential antigenicity in humans of murine MAbs [52]. The design of fusion proteins such as a

fusion of antibodies/antibody fragments with avidin (discussed in [25c]), could be a step beyond the chemical coupling approach described here. Some important issues in the design of drugs suitable for vector-mediated delivery have been touched in this review.

Finally, it should be pointed out that these three spheres are intersecting, and that success will only be possible by an interdisciplinary approach, integrating fields from organic chemistry and biochemistry, molecular biology and genetic engineering, to pharmacokinetics and transport physiology.

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