Targeting Neurotherapeutic Agents Through the Blood-Brain Barrier

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The blood-brain barrier (BBB) is maintained by the endothelial tight junctions within the brain microvasculature. Most small-molecule neuropharmaceutical agents and virtually all large-molecule drugs do not cross the BBB. The BBB problem is the rate-limiting factor preventing the transfer of progress in the molecular neurosciences to the development of clinically effective neurotherapeutic agents. The future development of neurotherapeutic agents will be accelerated by the development of BBB drug-targeting technology.

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THE BLOOD-BRAIN BARRIER AND NEUROTHERAPEUTIC AGENTS

Conventional neurotherapeutic agents produced by the pharmaceutical industry are nearly exclusively small-molecule drugs. Small-molecule drugs provide symptomatic relief for certain brain disorders, particularly the different types of epilepsy and affective disorders. However, many neurologic disorders (stroke, Alzheimer disease, the neurologic symptoms of acquired immunodeficiency syndrome, brain tumors, ataxias, and other genetic disorders) remain intractable to treatment with conventional small-molecule pharmaceutical agents.

Large-molecule drugs have the potential to be curative in patients with many neurologic disorders, but none of these large-molecule drugs cross the blood brain barrier (BBB). Although it is not widely recognized, more than 98% of small-molecule drugs do not cross the BBB.

Despite the importance of the BBB to the future development of neurotherapeutic agents, this area is underdeveloped within the neurosciences. To my knowledge, no pharmaceutical company in the world has a BBB drug delivery program! It is not unusual for an entire conference to be convened on a given neurologic disorder (eg, brain tumors), with no discussion devoted to the issue of targeting drugs through the BBB.

The lack of integration of BBB science within the overall neurotherapeutics field has become a rate-limiting factor in the translation of progress in the molecular neurosciences into the development of clinically effective neurotherapeutic agents.

If solutions to the BBB problem were found, then the number of neurotherapeutic agents available for clinical trials would increase by a log order of magnitude, including small- and large-molecule drugs. What is needed is the development of brain drug-targeting technology that enables the noninvasive delivery of small- or large-molecule drugs, including gene medicines, through the BBB of the human brain.

BRAIN DRUG-TARGETING TECHNOLOGY

The usual noninvasive approach to solving the brain drug delivery problem is to “lipidize” the drug, wherein the polar functional groups on the drug are masked with lipid groups. The water-soluble parts of the drug restrict BBB transport, and the masking of these molecular groups with lipid, and the conversion of a water-soluble drug into a lipid-soluble “prodrug,” is the traditional chemistry-driven solution to the BBB problem. However, there are few, if any, examples of drugs in modern neu-
Chimeric peptides are formed when a drug that is normally not transported through the BBB is conjugated to a brain drug-targeting vector. The latter is an endogenous peptide, modified protein, or peptidomimetic monoclonal antibody (MAb) that undergoes receptor-mediated transcytosis (RMT) through the BBB on endogenous receptor systems such as the insulin receptor or the TfR. Peptidomimetic MAb binds to exofacial epitopes on the BBB receptor that are removed from the endogenous ligand binding site and "piggyback" across the BBB on the endogenous RMT system within the BBB, without inhibition of transport of the endogenous ligand. The conjugation of drugs to brain drug transport vectors is facilitated with the use of avidin-biotin technology.

An alternative approach, which can be used for either small- or large-molecule drugs, is to reformulate the drug so that the molecule can access one of the many endogenous transport systems localized within the brain capillary endothelial wall, which forms the BBB in vivo.

There are 3 different classes of endogenous transport systems within the BBB: carrier-mediated transport systems, receptor-mediated transcytosis (RMT) systems, and active efflux transporters (AETs). The carrier-mediated transport systems include the glucose and amino acid carriers and mediate the bidirectional movement of small-molecule nutrients and vitamins between the blood and the brain. The RMT systems include the BBB insulin receptor and the transferrin receptor (TfR) and mediate the bidirectional movement of large-molecule peptides between the blood and the brain. These endogenous transporters are natural portals of entry to the brain of drugs that are formulated to enable binding and transport by these endogenous systems. Based on the knowledge that these endogenous transport systems exist, drugs may be reformulated to enable transport into the brain via the endogenous BBB transporters. For example, a monoamine drug may not cross the BBB. However, the neutral amino acid analog of this monoamine drug may cross the BBB on the endogenous neutral amino acid transporter. Once inside the brain, the amino acid analog may be decarboxylated to yield the original monoamine drug. The classic example of using an endogenous BBB transporter to solve the brain drug delivery problem is the use of levodopa to increase the cerebral dopamine level. Levodopa and dopamine are water soluble and would not normally cross the BBB. However, levodopa, a neutral amino acid, crosses the BBB via a large neutral amino acid transporter, the large neutral amino acid transporter 1 isoform. Apart from large neutral amino acid transporter 1, there are more than a dozen other endogenous carrier-mediated transport systems within the BBB that could be portals of entry for drugs that are appropriately designed to access these systems.

None of the drugs that constitute triple-drug therapy for the treatment of the acquired immunodeficiency syndrome cross the BBB, and the eradication of the human immunodeficiency virus in the brain of patients with the acquired immunodeficiency syndrome has not been possible. The protease inhibitors are substrates for P-glycoprotein, and zidovudine and lamivudine are substrates for other AET systems within the BBB. The molecular cloning of the AET systems could lead to the development of "codrugs" that inhibit the efflux transporter and thereby allow for increased brain penetration of drugs such as protease inhibitors, zidovudine, or lamivudine. Such codrugs would be used in conjunction with conventional drugs for treatment of the acquired immunodeficiency syndrome, just as aromatic amino acid decarboxylase inhibitors are coadministered with levodopa to increase brain uptake of the drug.

Whereas the carrier-mediated transport and AET systems are portals of entry for small-molecule drugs, large-molecule drugs and gene medicines may be delivered to the brain via the RMT systems, because these transport large-molecule endogenous peptide ligands. The development of large-molecule neurotherapeutic agents must be considered, because there is not a single chronic disease of the brain or other organs, apart from infectious diseases, that is cured by small-molecule drug therapy. The large-molecule drugs have the potential to be curative drugs or at least pharmaceutical agents that substantially alter the course of neurologic disease.

CHIMERIC PEPTIDE TECHNOLOGY

Chimeric peptides are formed when a drug that is normally not transported through the BBB is conjugated to a brain drug-targeting vector. The latter is an endogenous peptide, modified protein, or peptidomimetic monoclonal antibody (MAb) that undergoes receptor-mediated transcytosis (RMT) through the BBB on endogenous receptor systems such as the insulin receptor or the TfR. Peptidomimetic MAb binds to exofacial epitopes on the BBB receptor that are removed from the endogenous ligand binding site and "piggyback" across the BBB on the endogenous RMT system within the BBB, without inhibition of transport of the endogenous ligand. The conjugation of drugs to brain drug transport vectors is facilitated with the use of avidin-biotin technology. In this approach, a drug is monobiotinylated in parallel with the production of a vector/avidin or a vector/streptavidin (SA) fusion protein. The biotinylated drug is produced in one vial and the vector/avidin fusion protein is produced in another vial, and the 2 vials are mixed before administration. Owing to the extremely high affinity of avidin or SA binding of biotin, there is instantaneous capture of the biotinylated neurotherapeutic agent by the vector/avidin or vector/SA fusion protein. Monoclonal antibody/avidin and MAb/SA fusion genes and fusion proteins are produced with genetic engineering. Therefore, the conjugates of neurotherapeutic agents and brain drug transport vectors, also called chimeric peptides, are not overengineered biomolecules, but are drugs that can be produced with existing pharmaceutical technology.

A panel of species-specific brain drug delivery vectors has been developed. Brain drug delivery in rats is possible with the OX26 mouse MAB to the rat TfR. The OX26 MAB is not active in mice, and brain drug delivery in the mouse is enabled with the 8D3 rat MAb to the mouse TfR. Drug delivery in Old World primates is achieved with the use of the 83-14 mouse MAB to the human insulin receptor (HIR). Brain drug delivery in humans is possible with the genetically engineered chimeric HIR MAb. The activity of the genetically engineered chimeric HIR MAb is identical to that of the original murine 83-14 HIR MAb, and the chimeric antibody is avidly taken up by the primate brain, as demonstrated in the Figure.
and injected intravenously into the anesthetized rhesus monkey. The brain was scanned during a 2-hour period to yield the image shown in the Figure, A. The gray and white matter tracks are clearly delineated owing to the greater vascular density in gray matter relative to white matter. The brain uptake of the HIR MAb in the rhesus monkey is 2% to 4% of the injected dose, 15,16 which is a level of brain uptake that is 1 to 2 log orders greater than the brain uptake of a neuroactive small molecule such as morphine. 2 In contrast, there is no measurable brain uptake of an IgG molecule that does not react with a BBB RMT system. 15 The genetically engineered chimeric HIR MAb can be used in human clinical trials. Therefore, the application of large-molecule neurotherapeutic agents discussed later for preclinical studies in the rat could be extended to humans with existing technology.

A, A brain scan of a living rhesus monkey 2 hours after an intravenous injection of a genetically engineered chimeric monoclonal antibody (MAb) to the human insulin receptor that is radiolabeled with Indium In 111 (111In). There is widespread uptake of this blood-brain barrier (BBB) drug-targeting vector in the primate brain. 16 In contrast, there is no visible radioactivity in the brain following the administration of the isotype control antibody. 15 B, Top, Structure of the epidermal growth factor (EGF) chimeric peptide. The EGF is radiolabeled with diethylenetriamine pentaacetic acid (DTPA) that chelates the 111In. The EGF is biotinylated with a 200-atom linker composed of a 3400-d polyethylene glycol (PEG), designated PEG3400. The EGF, PEG, and biotin complex is captured by a conjugate of streptavidin (SA) and the OX26 MAb to the rat transferrin receptor (TfR). Bottom, Autopsy stains are shown in panels 1 and 3, and in vivo brain scan results are shown in panels 2 and 4. The autopsy sections were stained immunocytochemically using an MAb to the human EGF receptor (EGF-R), which demonstrates expression of the EGF-R in the experimental U87 human glioma cells in nude rat brain specimens. There is no imaging of the brain tumor when the EGF peptide radiopharmaceutical is administered alone (panel 4) because the EGF does not cross the BBB. In contrast, the brain tumor is imaged with the EGF chimeric peptide that can undergo transport across the BBB in vivo (panel 2). Data from Kurihara and Pardridge. 17 C, Top, Structure of the brain-derived neurotrophic factor (BDNF) chimeric peptide. The BDNF is conjugated with PEG2000 and is bound via a biotin linker to a conjugate of SA and the OX26 MAb to the rat TfR. The SA and the OX26 MAb are attached via a thioether (-S-) linkage. This bifunctional molecule may bind the endothelial TfR, to cause transport through the BBB, and the neuronal trkB receptor, to mediate neuroprotection. Bottom, Coronal sections of rat brain stained with 2% 2,3,5-triphenyltetrazolium chloride. There were 4 treatment groups and 4 rats per group, and a coronal section for each rat is shown. The rats were subjected to permanent middle cerebral artery occlusion and treated intravenously with isotonic sodium chloride, BDNF alone, TfR MAb alone, or the BDNF-MAb conjugate. The volume of the infarcted brain, which appears purple, is reduced 65% by delayed intravenous administration of the BDNF-MAb conjugate. Data from Zhang and Pardridge. 18 D, Rats were subjected to 10 minutes of transient forebrain ischemia, resuscitated, and then humanely killed 7 days later. The pyramidal neuron density in the CA1 sector of the hippocampus was examined with Nissl staining of rat brain sections. The Nissl stains of the animals treated with BDNF alone are shown in panels 1 and 2, and the Nissl stains of the animals treated with the BDNF-MAb conjugate are shown in panels 3 and 4. Whereas the BDNF alone results in no neuroprotection in this global ischemia model, there was complete normalization of the pyramidal cell density in the CA1 sector of the hippocampus in the animals treated with BDNF chimeric peptide. The CA1 area of the hippocampus at either low or high magnification is shown by the arrows. Data from Wu and Pardridge. 19
NEUROIMAGING WITH PEPTIDE RADIOPHARMACEUTICALS

The practice of brain imaging uses small-molecule radiochemicals that bind to monoamine or amino acid neurotransmitter systems. Whereas there are less than a dozen monoaminergic or amino acidergic neurotransmitter systems, there are hundreds of peptidergic neurotransmitter systems. Therefore, the use of peptide radiopharmaceuticals could greatly increase the diagnostic potential of neuroimaging technology. Potential candidates for neuroimaging include epidermal growth factor (EGF) peptide radiopharmaceuticals for the early detection of brain tumors and Aβ peptide radiopharmaceuticals as a diagnostic brain scan for Alzheimer disease.

Many malignant gliomas overexpress the EGF receptor (EGF-R), and EGF is a potential peptide radiopharmaceutical for the imaging of brain tumors should this large molecule be made transportable through the BBB. The top of the Figure, B, illustrates the molecular reformulation of attachment of a radionuclide (111In) and conjugation to a BBB drug delivery system, composed of the OX26 MAb to the rat TfR. One lysine group on the EGF was biotinylated via an extended 200-atom linker composed of a 3400-d polyethylene glycol. The EGF, polyethylene glycol, and biotin complex were immediately captured by a conjugate of the TfR MAb (OX26) and SA. This bifunctional chimeric peptide binds the BBB TfR, to mediate transport across the BBB, and the EGF-R, to cause sequestration within the brain tumor region beyond the BBB. U87 human glioma cells were implanted in the caudate putamen nucleus of nude rats, and experimental human gliomas developed during the next 15 days. Autopsy sections of the brain were stained with an MAb to the EGF-R, and the immunocytochemistry results demonstrated the development of brain tumors in the implanted region that expressed the immunoreactive EGF-R, as shown in panels 1 and 3 of the Figure, B. Before humane killing, these tumor-bearing animals were administered either 111In-EGF alone or the 111In-EGF peptide radiopharmaceutical conjugated to the TfR MAb. The peptide radiopharmaceutical formulations were administered intravenously, and brain scans were obtained during the next 2-hour period before humane killing of the animal. The brain tumor was not imaged by the EGF alone, owing to the lack of transport of the EGF through the BBB (Figure, B, panel 4). In contrast, the brain tumor was imaged with the EGF peptide radiopharmaceutical that was conjugated to the BBB drug-targeting system (Figure, B, panel 2). There was a 20-fold enrichment in radioactivity over the brain tumor relative to a normal brain. Based on this high tumor-brain ratio, the EGF chimeric peptide may also be useful as a neurotherapeutic agent for the radiotherapy of brain tumors.

The substitution of the 111In radionuclide with gadolinium would result in the development of a peptide magnetopharmaceutical that could be used with magnetic resonance imaging–based brain scans, and this could facilitate the detection of a residual glioma by intraoperative magnetic resonance imaging. Apart from tumors, other structures in the brain and other neurologic diseases could be imaged with peptide radiopharmaceuticals or peptide magnetopharmaceuticals once these large-molecule drugs can cross the BBB with the use of brain drug-targeting technology.

PROTEIN NEUROTHERAPEUTIC AGENTS AND NEUROPROTECTION IN STROKE

Virtually all small-molecule neuroprotective agents have failed in clinical stroke trials because either (a) these molecules have unfavorable safety profiles or (b) the drugs do not cross the BBB. The therapeutic window for neuroprotection is the first 3 hours after stroke, and during this time, the BBB is intact. The BBB is disrupted in later stages following stroke, but at this time, chances for neuroprotection have been lost. Therefore, if effective neuroprotective agents for stroke are to be developed, these molecules must have favorable safety profiles and must be able to cross the BBB. There are more than 30 neurotrophins in the brain, and many of these naturally occurring neuropharmaceutical agents are neuroprotective in animals subjected to experimental stroke when injected directly into the brain. However, the neurotrophins are ineffective neuropharmaceutical agents following intravenous administration because these molecules do not cross the BBB. A model neurotrophin, brain-derived neurotrophic factor (BDNF), was formulated to enable BBB transport, and the BDNF chimeric peptide is neuroprotective following delayed intravenous administration in either regional or global brain ischemia.

Regional Brain Ischemia

The structure of the BDNF chimeric peptide is shown at the top of the Figure, C. The BDNF chimeric peptide is a bifunctional molecule, which binds the BBB TfR, to enable transport into the brain, and the neuronal trkB receptor, to enable neuroprotection once in the brain. Regional ischemia was induced with the middle cerebral artery occlusion (MCAO) model. Brain-derived neurotrophic factor chimeric peptides are neuroprotective in the permanent MCAO model, as shown in the Figure, C. Adult rats subjected to permanent MCAO were treated intravenously with unconjugated BDNF, unconjugated TfR MAb, or the BDNF-MAb conjugate, and only the BDNF chimeric peptide caused a reduction in stroke volume at 24 hours after the infarction. The BDNF-MAb conjugate was administered intravenously at a dose of 1, 5, and 50 µg of BDNF per rat. These doses decrease the infarct volume by 6% (P<.05), 43% (P<.01), and 65% (P<.01), respectively. Significant reduction of stroke volume was still observed if the administration of the BDNF-MAb conjugate was delayed for 1 to 2 hours after permanent MCAO, indicating a therapeutic window of approximately 2 hours. The reduction in stroke volume is apparent on inspection of the coronal sections of brain stained with 2% 2,3,5-triphenyltetrazolium chloride, as shown in the bottom of the Figure, C. The mean±SD
hemispheric stroke volume in the animals treated with isotonic sodium chloride was $350 \pm 21 \text{ mm}^3$, and there was no reduction in this stroke volume in the animals treated with either MAb or BDNF alone.\(^\text{18}\) However, the mean$\pm$SD stroke volume was reduced 65% to $121 \pm 23 \text{ mm}^3$ with the intravenous administration of BDNF-MAb conjugate at a dose of 50 µg per rat. The neuroprotective effects of BDNF chimeric peptides in rats subjected to regional brain ischemia are replicated with a 1-hour reversible MCAO model.\(^\text{31}\) In this series of experiments, the delayed intravenous administration of unconjugated BDNF resulted in no neuroprotection in brain specimens analyzed at either 24 hours or 7 days after 1-hour MCAO. In contrast, there was a 68% and a 70% reduction in cortical stroke volume at 24 hours and 7 days, respectively, after the intravenous administration of 50 µg of the BDNF conjugate per rat in the 1-hour reversible MCAO model.\(^\text{31}\) These results indicate that BDNF could be used in the treatment of acute stroke as a novel neuroprotective agent, should the drug be made transportable through the BBB. Another potential application of neurotrophin chimeric peptides is the treatment of global brain ischemia, such as following a cardiac arrest or a severe hypotensive episode.

**Global Brain Ischemia**

Global brain ischemia was induced in adult rats with the transient forebrain ischemia model.\(^\text{33}\) The electroencephalogram was made isoelectric for a 10- to 12-minute period with hypotension and bilateral common carotid artery occlusion.\(^\text{19}\) After the period of isoelectric electroencephalography, the rats were resuscitated and treated intravenously with 50 µg of unconjugated MAb, unconjugated BDNF, or a conjugate of BDNF and the OX26 TfR MAb. Following treatment and recovery, the animals were humanely killed 7 days later for brain sectioning and Nissl staining, as shown in the Figure, D. Quantitative counting of pyramidal neurons in the hippocampal CA1 sector showed that there was a mean$\pm$SD 68% $\pm$ 10% decrease in hippocampal CA1 neuronal density in the rats subjected to transient forebrain ischemia and that there was no therapeutic effect following the intravenous administration of BDNF or MAB alone.\(^\text{19}\) The Nissl stains of brain specimens from rats treated with BDNF alone are shown in panels 1 and 2 of the Figure, D. This study shows a loss of neurons in the CA1 sector of the hippocampus (Figure, D, panel 2). Shrunken dead neurons in this region of the hippocampus are visible at high magnification (Figure, D, panel 1). In contrast, there was no loss of CA1 sector pyramidal neuron density in rats treated with the BDNF-MAB conjugate (Figure, D, panels 3 and 4). These studies demonstrate that BDNF is neuroprotective in regional and global ischemia,\(^\text{18,19,31}\) providing the neurotrophin is conjugated to a BBB drug-targeting system. Similarly, there are many other neurotrophins that could be used as protein neurotherapeutic agents after reformulation of the protein to enable transport across the BBB.\(^\text{3}\) Neurotrophin chimeric peptides could be used in the short-term treatment of stroke or brain trauma or in the treatment of chronic neurodegenerative diseases.

**BBB GENOMICS**

The future development of brain drug-targeting technology will be facilitated by the ongoing discovery of tissuespecific gene expression at the brain capillary endothelial cell, which forms the BBB in vivo. The discovery of BBB-specific gene expression will enable the production of brain-specific drug-targeting vectors. The identification of BBB-specific genes is accelerated with a BBB genomics program based on gene microarray technology.\(^\text{3}\) A polymerase chain reaction–based subtraction cloning method, suppression subtractive hybridization, was used to produce rat brain capillary complementary DNA, and this tester complementary DNA was subtracted with driver complementary DNA produced from messenger RNA isolated from rat liver and rat kidney.\(^\text{34}\) Screening just 5% of the BBB complementary DNA library resulted in the identification of 50 gene products, and more than 80% of these were selectively expressed at the BBB based on Northern blot analysis.\(^\text{34}\) These BBB selective genes included novel gene sequences not found in existing databases, expressed sequence tags, and known genes that were not previously shown to be selectively expressed at the BBB. Genes in this latter category include tissue plasminogen activator, insulin-like growth factor II, the PC-3 gene product, myelin basic protein, regulator of G-protein signaling 5, utrophin, IκB, connexin 43, the class I major histocompatibility complex, the rat homologue of the transcription factors hbrm or EZH1, and organic anion–transporting polypeptide type 2. The identification of tissue-specific gene expression at the BBB using a brain vascular genomics program will lead to future discovery of new targets for brain drug delivery, and may also elucidate mechanisms of brain pathophysiology at the microvascular level.

**CONCLUSIONS**

Neurotherapeutics needs to expand from the 20th-century base of chemistry-driven small-molecule drug discovery. Small molecules are largely palliative medicines with often unfavorable safety profiles. Functional genomics will create the platform for future biology-driven large-molecule drug discovery. However, large molecules do not cross the BBB. Therefore, large molecules, which have the potential to cure neurologic disease, will not become clinically effective neurotherapeutic agents without the development of brain drug-targeting technology. The number of large-molecule pharmaceutical agents that could be used to treat neurologic disease will expand with the continued application of functional genomics and the discovery of novel secreted brain proteins. Future advances in the molecular neurosciences may lessen the singular reliance on lipid-soluble smallmolecule drugs. The future convergence of functional genomics and large-molecule neurotherapeutic agents is possible, but this will require the development of brain drug-targeting technology.

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