



## Diphtheria toxin receptor-targeted brain drug delivery

Pieter J. Gaillard<sup>a,\*</sup>, Arjen Brink<sup>a</sup>, Albertus G. de Boer<sup>b</sup>

<sup>a</sup>*to-BBB technologies BV, BioScience park Leiden, Gorlaeus Laboratories, LACDR-F'col, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

<sup>b</sup>*Blood–Brain Barrier Research Group, Division of Pharmacology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, The Netherlands*

**Abstract.** Brain drug delivery is limited by the blood–brain barrier (BBB). We have newly identified the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), which is also known as the diphtheria toxin receptor (DTR), as a well characterized internalizing transport receptor on the BBB for the targeting of drugs to the brain. This transport receptor has several unique advantages. It makes use of a non-toxic endogenous transport mechanism called receptor-mediated endocytosis, with proven cargo-carrying properties across the BBB (e.g., brain delivery (transcytosis) of large proteins and liposomes containing drugs); the receptor has no endogenous ligands and thus neither competition from endogenous ligands, nor blockade of transport to the brain of essential nutrients is to be expected; the membrane bound receptor is constitutively expressed on the BBB, neurons, and glial cells; receptor expression is highly amplified in disease conditions and thus allows for site-specific disease targeting; and the biological activity of the receptor can be modulated by a variety of pharmacologically active compounds (like heparin and proteinase inhibitors). Furthermore, the targeting technology makes use of a non-toxic mutant of diphtheria toxin (known as CRM197) as the receptor-specific carrier protein. This carrier protein has several unique advantages as well. It is a well characterized protein (i.e., known receptor binding domain, conjugation sites, manufacturing process), and it has already been successfully marketed for human use to millions of people in vaccination programs, and recently also in anti-cancer trials, with a proven carrier efficacy and excellent safety profile. We have been able to demonstrate proof-of-principle data in our cell culture model of the BBB, as well as in guinea pigs with this novel brain drug targeting technology, including: functional expression of DTR; safety of CRM197 carrier protein; transport efficacy of CRM197 carrier protein conjugated directly to horseradish peroxidase (HRP, serving as a 40 kDa ‘model’ protein drug); and specific *in vivo* brain uptake of DTR-targeted HRP. In conclusion, the

\* Corresponding author. Tel.: +31 715276268; fax: +31 848709728.  
E-mail address: [gaillard@toBBB.com](mailto:gaillard@toBBB.com) (P.J. Gaillard).

DTR seems to be a human applicable, safe, and effective uptake receptor for the targeting of drugs to the brain. © 2005 Elsevier B.V. All rights reserved.

**Keywords:** Blood–brain barrier; Heparin-binding epidermal growth factor; Diphtheria toxin receptor; Receptor-mediated endocytosis; Drug targeting

---

## 1. Introduction

Brain drug delivery is limited by the blood–brain barrier (BBB). Particularly, hydrophilic and large drugs (e.g., biopharmaceuticals) poorly pass the blood–brain barrier and generally do not reach the brain in sufficient concentrations to be effective. As a result, many high potential ‘would be’ central nervous system (CNS) drugs (especially biopharmaceuticals) are currently not available to the brain [1]. Unlike small molecules, large biopharmaceutical drugs are unlikely candidates for chemical modifications to enhance their permeability across the blood–brain barrier. Invasive and potentially harmful technologies to patients, like direct stereotactic injections, intrathecal infusions, and blood–brain barrier disruption, are now being evaluated in clinical settings. Because of the severe neurological consequences, these techniques are only allowed to be applied in selected life-threatening diseases. Moreover, these technologies are far from effective in delivering drugs throughout the whole brain. Therefore, there is an established need to improve the delivery of biopharmaceutical drugs to the brain.

Since almost every neuron is perfused by its own capillary, the most effective way of delivering biopharmaceutical drugs is achieved by targeting to endogenous transport receptors on these capillaries. In fact, the total length of capillaries in the human brain is impressive (~600 km) with a large surface area (~20 m<sup>2</sup>) for effective exchange of drugs [2]. An intravenous (injectable) drug delivery technology for CNS-active biopharmaceutical drugs will allow for the (enhanced) treatment of many more brain disorders. In many preclinical studies, the insulin- or (melano)transferrin receptors have been successfully utilized for this purpose [2]. Currently, there are, however, no such brain drug delivery technologies on the market for human use. One reason for this might be that these technologies still involve potential safety hazards, like the obstruction of brain entry of essential compounds (like insulin or iron), or potentially dangerous interactions with endogenous substrates (as was published for melanotransferrin/p97 which seems to activate plasminogen in rats [3]). Therefore, there still exists a need for new, safe, and effective brain drug delivery technologies that can potentially be applied to humans.

In this paper, we describe the use of the receptor for diphtheria toxin (DTR), the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), as a new endogenous transport receptor for the delivery of drugs across the BBB. Specifically, we used CRM197, a non-toxic mutant protein of diphtheria toxin, as the receptor-specific carrier protein. CRM197 has already been used as a safe and effective carrier protein in human vaccines for a long time [4], as well as in recent anti-cancer trials [5]. This resulted in a large body of prior knowledge on the carrier protein, including its transport receptor and mechanism of action, receptor binding domain, conjugation- and manufacturing process, and kinetic- and safety profile both in animals

and humans. Diphtheria toxin (and CRM197) enters the cell by a well-characterized, safe, and effective mechanism called receptor-mediated endocytosis [6], alike transferrin and p97. Membrane-bound HB-EGF is constitutively expressed on the BBB, neurons, and glial cells. Moreover, its expression on the BBB is strongly upregulated under disease conditions (such as stroke, brain tumors, and seizures) and its biological activity can also be modulated by a variety of pharmacologically active compounds (like heparin and proteinase inhibitors) [7–10].

By means of our dynamic cell culture model of the BBB, we were able to demonstrate the functional expression of the DTR, safety of the CRM197 carrier protein, and transport efficacy of CRM197 carrier protein conjugates to a 40 kDa enzyme (horseradish peroxidase, HRP, serving as a ‘model’ protein drug). In addition, we have demonstrated proof-of-principle with this novel brain drug targeting technology by the specific brain uptake of DTR-targeted HRP in guinea pigs *in vivo*. Collectively, these results indicate that CRM197 may indeed be developed into a new, safe, and effective brain drug delivery carrier protein for human applications.

## 2. Methods

### 2.1. Protein assays

Proteins (diphtheria toxin (DT), CRM197, holo-transferrin (Tf), and bovine serum albumin (BSA) from Sigma (Zwijndrecht, The Netherlands)) were conjugated to HRP by means of a HRP conjugation kit (directed at primary amine groups) according to the manufacturers instructions (Alpha Diagnostic International, San Antonio, TX, USA), as described before (Visser et al., 2004, *J Drug Target.* 12 (3): 145–150). In addition, conjugated proteins (1:1 weight/weight ratio) were further purified on a HiPrep 16/60 column packed with Sephadryl S-200 HR matrix (Amersham Biosciences, UK).

HRP activity and protein contents were detected using standard colorimetric assays with the appropriate calibration curves, as described before [11]. Briefly, 20 µl of appropriately diluted HRP samples were incubated with TMB liquid substrate for 20 min, after which 0.5 M H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. HRP (0–2 ng/ml) was used for a standard curve and absorption was read at 450 nm. Cellular protein contents were determined using Biorad DC reagents and BSA for a standard curve (0–400 µg/ml). Absorption was read at 690 nm.

### 2.2. Cell culture experiments

The preparation and characterization of our cell culture model of the BBB has been described in great detail before [12]. Briefly, brain capillaries were isolated from cortices of brains of bovine origin, obtained at the slaughterhouse. Astrocytes were isolated from cortices of brains of newborn Wistar rats and used to prepare astrocyte-conditioned medium and co-cultures. Brain capillary endothelial cells (BCEC) were cultured from brain capillaries on collagen and fibronectin coated culture flasks in 50% astrocyte-conditioned medium. BCEC were passaged into collagen coated 96-well plates or Transwell polycarbonate filters and cultured to tight confluent monolayers. For BCEC-astrocyte cocultures, astrocytes were seeded on the bottom of the filters 2 days before BCEC.

After apical exposure of BCEC cocultured with astrocytes on filters to DT, CRM197, human recombinant soluble HB-EGF (from R and D Systems Europe Ltd., UK), or the HRP-conjugated proteins, BBB functionality was assessed in time by monitoring changes in transendothelial electrical resistance (TEER) across the filters using an electrical resistance system (ERS) with a current-passing and voltage-measuring electrode (Millicell-ERS, Millipore Corporation, Bedford, MA, USA). TEER ( $\Omega \cdot \text{cm}^2$ ) was calculated from the displayed electrical resistance on the readout screen by subtraction of the electrical resistance of a collagen coated filter without cells and a correction for filter surface area. TEER across collagen coated filters with only astrocytes on the bottom was close to zero.

BCEC in 96-well plates were incubated with HRP-conjugated proteins corresponding to a concentration of 5  $\mu\text{g}/\text{ml}$  of un-conjugated HRP for 1–4 h. BCEC were lysed after thorough washing with PBS by 40  $\mu\text{l}$  of an aqueous solution of 0.1% Na-deoxycholate for determination of HRP activity or cellular protein.

For the assessment of the transcytosis of HRP-conjugated proteins, the BCEC-astrocyte cocultures were treated with 312.5  $\mu\text{M}$  8-(4-chlorophenylthio (CPT))-cAMP, and 17.5  $\mu\text{M}$  RO-20-1724 in complete HEPES buffered medium with serum for the last 2 days in order to dramatically increase tightness of (i.e., reduce paracellular leakiness) of the in vitro BBB [12], both at 37 °C and at 4 °C. The average TEER across BCEC-astrocyte cocultures increased from  $149.8 \pm 5.4 \Omega \cdot \text{cm}^2$  (mean  $\pm$  standard error,  $n=18$ ) to  $834 \pm 77 \Omega \cdot \text{cm}^2$  (mean  $\pm$  standard error,  $n=24$ ) after treatment with 8-4-CPT-cAMP and RO-20-1724. No difference in DT sensitivity was observed between cells un-treated and cells treated as such (data not shown). For the 4 °C arm of the experiment, filters were allowed to cool down in the refrigerator for 1 h before the transport experiment was started. HRP-conjugated proteins were added to the apical side of the filter insert and the filter was directly transferred into a fresh well containing 250  $\mu\text{l}$  fresh medium. Every hour, up to 4 h in total, this procedure was repeated in order to prevent possible re-endocytosis of HRP-conjugated proteins by the abluminal side of the BCEC. Cumulated HRP activity of transcytosed HRP into the basolateral compartment was determined.

### *2.3. In vivo animal studies*

Brain uptake of DT or Tf conjugated to HRP was determined 1.5 h (which is known to be well before any toxic effects of DT are observed) after an intravascular bolus injection of the conjugates (corresponding to a concentration of 500  $\mu\text{g}/\text{ml}$  in 0.5 ml saline of un-conjugated HRP), and compared to an equal concentration of free HRP, in young male Guinea pigs (Dunkin-Hartley HsdPoc:HD, 250–300 g). Briefly, the animals were anesthetized with isoflurane inhalation (4% induction, 1–1.5% maintenance) in an air/oxygen mixture (2:1). A cannula was placed in the carotid artery for blood sample collection and drug administration. At 1.5 h after injection of the proteins, animals were deeply anesthetized with 4% isoflurane (1–2 min) and subsequently the whole animal (including the brain) was perfused with saline via the cardiac aorta (<5 min), to clear the blood vessels from blood. Directly after, animals were decapitated and brains were removed from the skull for further analysis. Only brains that were cleared from all blood (based on a visual inspection of the brain) were used for further analysis. Central cross-

sections (ear to ear) of about 0.5 cm of the perfused brains (and one non-perfused control brain (i.e., no injections of HRP or HRP-conjugates)) were dissected and were directly snap-frozen in isopentane and stored until use at minus 80 °C.

Tissue sections were cut into 14 µm cryo-sections on a cryostat. Some sections were air fixed and HRP (or endogenous peroxidases in case of the non-perfused control brain) activity was stained directly by TMB (peroxidase substrate kit TMB, Vector Laboratories) for 30 min, washed in demi water for 5 min, and dehydrated in a series of ethanol and xylene (90% ethanol 2×1 min; 100% ethanol 2×1 min; xylene 2×1 min), and finally embedded in Entellan (Merck). No counterstaining was performed.

Other sections were fixed in 4% paraformaldehyde (15 min), and DT distribution in the brain was stained by immunohistochemistry for DT by mouse-anti-DT 1:10 (OBT0746, ImmunologicalsDirect.com), and a secondary HRP-goat-anti-mouse antibody 1:250 (Jackson ImmunoResearch). This primary antibody was able to selectively stain DT as well as DT-HRP conjugates in dot-blot pilot experiments, both on pure proteins samples and in the DT-HRP conjugated homogenate samples (data not shown). Endogenous peroxidases were blocked (20 min in PBS with 0.3% H<sub>2</sub>O<sub>2</sub> in 0.1% NaN<sub>3</sub>), and non-specific staining was prevented by 5% normal goat serum. HRP activity of the secondary antibody was stained by TMB (peroxidase substrate kit TMB, Vector Laboratories) for 10 min, washed in demi water for 5 min, and dehydrated in a series of ethanol and xylene (90% ethanol 2×1 min; 100% ethanol 2×1 min; xylene 2×1 min), and finally embedded in Entellan (Merck). No counterstaining was performed.

### 3. Results

#### 3.1. Functional expression of DTR on the BBB *in vitro*

After apical exposure to 1 ng/ml up to 10 µg/ml of DT, the TEER across BCEC-astrocyte cocultures decreased in a concentration- and time-dependent manner, while concentrations as low as 1 ng/ml were toxic after an overnight incubation period (Fig. 1A). These results indicate that DT is effectively taken up from the apical site (i.e., blood site) by BCEC in which it can exert its toxic effects.

After apical exposure to 100 ng/ml DT which was preincubated with soluble HB-EGF (1 h at room temperature), acting as a non-competitive antagonist for the DTR by binding to the receptor-binding domain of DT, the toxic effect of DT on BCEC-astrocyte cocultures decreased in a concentration dependent manner (Fig. 1B). In fact, a preincubation of 100 ng/ml DT with 10 microgram/ml of soluble HB-EGF completely prevented the DT-induced toxic effect on BCEC, even after an overnight assessment. These results indicate that DT-uptake in BCEC is effectively blocked by previous specific binding of DT to its soluble receptor, making it unable to exert its toxic effects within the BCEC.

After BCEC were preincubated with CRM197, the non-toxic mutant protein of DT, acting as a competitive antagonist at the DTR by binding to the receptor-binding domain for DT, the toxic effect of apical exposure to 100 ng/ml DT on BCEC-astrocyte cocultures was reduced (Fig. 1C). These results indicate that DT-uptake in BCEC is effectively

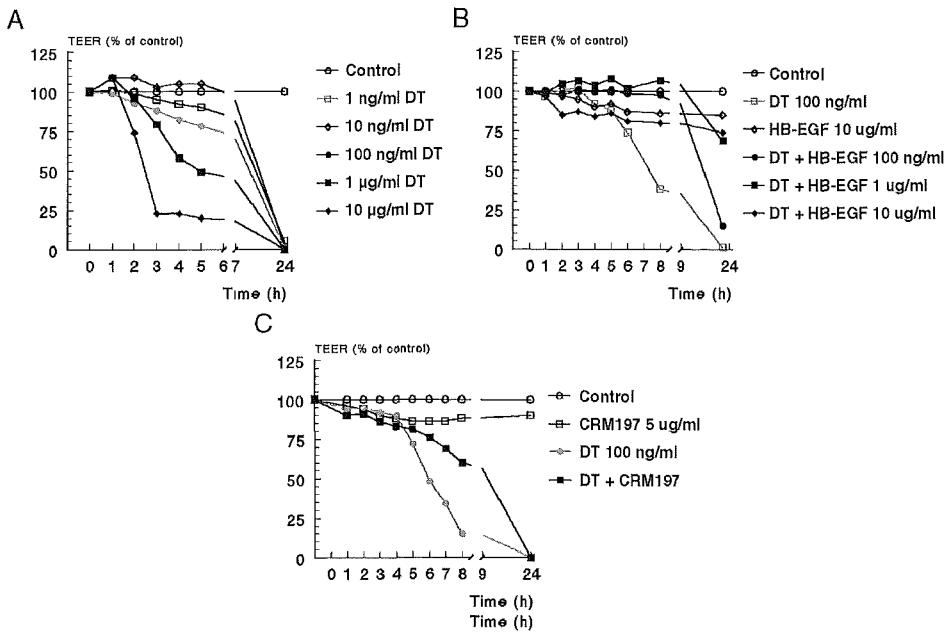


Fig. 1. Panel A is a diagram showing the effect on TEER (expressed as mean% of control) across BCEC-astrocyte cocultures apically exposed to various concentrations (1 ng/ml up to 10 µg/ml) of DT. Panel B is a diagram showing the effect of 100 ng/ml DT which was preincubated with various concentrations of soluble HB-EGF (0.1–10 µg/ml) before it was exposed to the apical side of the filter. Panel C is a diagram showing the effect of 100 ng/ml DT on cells that were pretreated for 1 h with 5 µg/ml CRM197.

antagonized by previous specific binding of CRM197 to the DTR, making it less available for DT to exert its toxic effects within the BCEC. Also note the absence of toxic effects of CRM197 on the BCEC-astrocyte cocultures (Fig. 1C), which was consistent up to the highest tested concentration of 50 µg/ml (data not shown).

### 3.2. Transport efficacy and specificity of HRP-conjugated CRM197 *in vitro*

After BCEC cultured as monolayers in 96-wells plates were incubated with HRP-conjugated proteins, the CRM197–HRP conjugate was preferably taken up by the BCEC when compared to BSA- and transferrin–HRP conjugates (Fig. 2A). These results indicate that CRM197 conjugated to a cargo of 40 kDa is specifically taken up by BCEC. After BCEC were incubated with CRM197–HRP-conjugate which was preincubated with 10 µg/ml soluble HB-EGF, the specific uptake of the CRM197–HRP conjugate was completely inhibited, as compared to the a-specific uptake of BSA–HRP-conjugate (Fig. 2B). These results indicate that CRM197 conjugated to a cargo of 40 kDa is specifically taken up by BCEC via a DTR-mediated uptake process.

After BCEC-astrocyte cocultures were incubated with HRP-conjugated proteins, the CRM197–HRP conjugate was preferably transcytosed across the BCEC when compared to BSA–HRP conjugates (Fig. 3). At 4 °C, the level of transport for the CRM197–HRP conjugate was identical to the BSA–HRP conjugate at 37 °C and 4 °C (Fig. 3). These

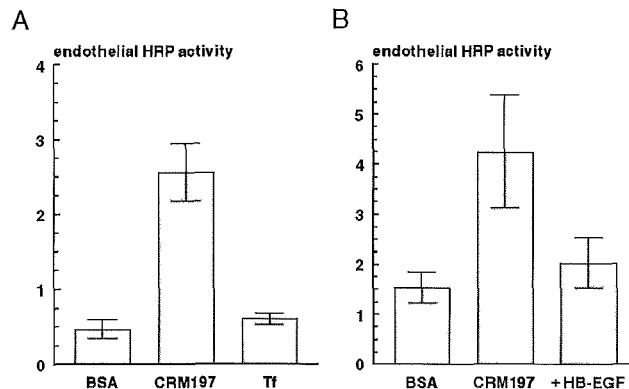


Fig. 2. Panel A is a diagram showing HRP activity in BCEC lysates (ng/ml, mean $\pm$ standard error,  $n=6$ ) after exposure to HRP-conjugated proteins (BSA, CRM197, and Tf). Panel B is a diagram showing HRP activity in BCEC lysates after exposure to HRP-conjugated BSA, HRP-conjugated CRM197, and HRP-conjugated CRM197 which was preincubated with 10  $\mu$ g/ml soluble HB-EGF.

results indicate that CRM197, even when conjugated to a protein cargo of 40 kDa, is specifically and actively transcytosed across the BBB.

### 3.3. Transport efficacy and specificity of HRP-conjugated CRM197 *in vivo*

For the assessment of the brain drug targeting ability via DTR *in vivo*, we used guinea pigs that were intravascularly injected with proteins (DT and Tf) conjugated to HRP, and free HRP. After the cryo-sections of the non-perfused control brain was directly stained for endogenous peroxidase activity by TMB, a distinct and strong staining pattern characteristic for blood vessels was observed throughout the whole section. A typical example for this pattern is shown in Fig. 4A. As can be appreciated from Fig. 4B, the perfusion procedure with saline via the cardiac aorta was able to completely remove this endogenous peroxidase activity. The TMB-stained cryo-sections of the well-perfused brains of free HRP injected animals showed, like the well-perfused control brain (not shown), no visible

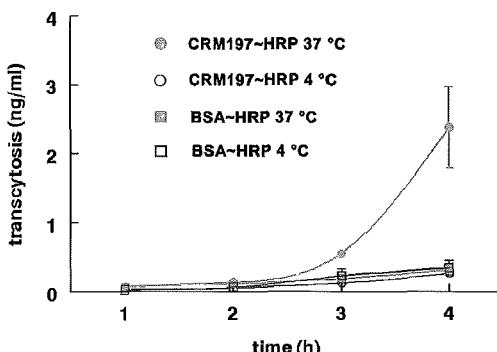
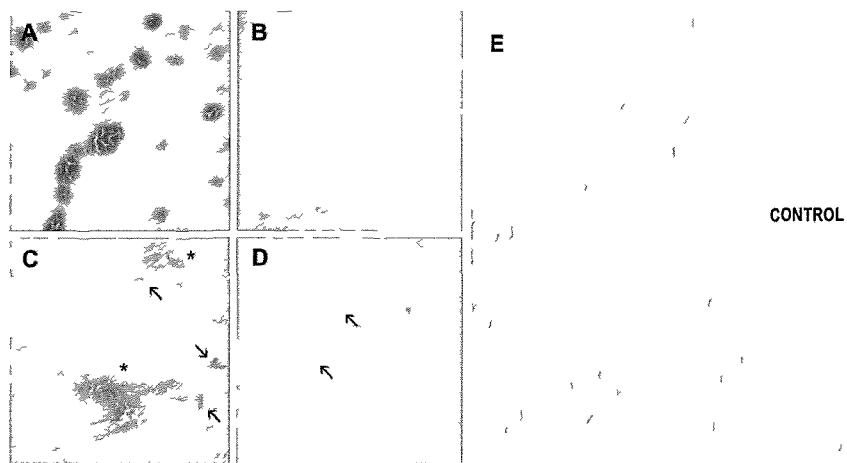


Fig. 3. Active and selective accumulated transcytosis of HRP-conjugated CRM197 (mean $\pm$ standard error of mean,  $n=6$  per group) across the *in vitro* BBB (lines with circles), when compared to HRP-conjugated BSA (lines with squares), at 37 °C (lines with filled symbols) and 4 °C (lines with open symbols).



**Fig. 4** Representative photographs of (A) a non-perfused control brain that was directly stained for endogenous peroxidase activity by TMB (note the distinct and strong staining pattern characteristic for blood vessels throughout the whole section); (B) TMB-stained cryo-sections of well-perfused brains of a free HRP injected animal (note that the perfusion procedure with saline via the cardiac aorta was able to completely remove the endogenous peroxidase activity seen in panel A), (C) TMB-stained cryo-sections of a well-perfused brain of a DT-HRP conjugate injected animal (note the staining patterns characteristic for association with small blood vessels (indicated by arrows), as well as the distinct staining areas characteristic for extravasated (i.e., transported) HRP across the blood vessels (indicated by asterisks)), (D) TMB-stained cryo-sections of a well-perfused brain of a Tf-HRP conjugate injected animal (note the few (if any) very faint staining patterns characteristic for association with small blood vessels (indicated by arrows)), (E) a cryo-section of a DT-HRP conjugate injected animal stained for DT by immunohistochemistry for DT by mouse-anti-DT (note the faint homogeneously distributed staining pattern throughout the whole section, magnification 100 $\times$ ), (panel E insert) a cryo-section of a free HRP injected animal stained for DT by immunohistochemistry for DT by mouse-anti-DT (note that the staining pattern was absent, magnification 100 $\times$ ) All magnifications in panels A, B, C, and D of the brain cortex cryo-sections are 40 $\times$

staining (Fig. 4B shows a representative photograph). The TMB-stained cryo-sections of the well-perfused brains of DT-HRP conjugate injected animals showed, however, staining patterns characteristic for association with small blood vessels (Fig. 4C shows a representative photograph indicated by arrows). In addition, several distinct staining areas throughout the whole section characteristic for extravasated (i.e., transported) HRP across the blood vessels were observed in these animals (Fig. 4C shows a representative photograph indicated by asterisks). In contrast, the TMB-stained cryo-sections of the well-perfused brains of Tf-HRP conjugate injected animals showed a few (if any) very faint staining patterns characteristic for association with small blood vessels (Fig. 4D shows a representative photograph indicated by arrows). Collectively, these results indicate that DT conjugated to a cargo of 40 kDa (i.e., HRP) is specifically taken up in the brain cortex (via the DTR), where free HRP and HRP conjugated to Tf are not.

Cryo-sections in which the DT distribution in the brain was stained by immunohistochemistry for DT by mouse-anti-DT showed a faint homogeneously distributed pattern throughout the whole section (Fig. 4E). This staining pattern was not observed in the free HRP (Fig. 4, insert in panel E marked 'control') and Tf-HRP conjugate (not shown)

injected animals. Collectively, these results indicate that DT (cleaved or still conjugated to HRP) is taken up in the brain.

#### 4. Discussion

In this paper, we clearly demonstrated that DT is very effectively taken up by BCEC, specifically via the DTR. The finding that the DTR is functionally expressed on the cells that constitute the BBB offers new opportunities to specifically target drugs to and across the BBB. In fact, any ligand that specifically binds to the DTR (like DT and CRM197) may prove useful for this purpose. We have effectively demonstrated that DTR-targeted delivery of HRP, serving as a ‘model’ protein drug, is indeed specifically targeted to and across the BBB.

The general concept of the use of protein toxins (or non-toxic derivatives thereof) as carriers for e.g., peptides and proteins, across membranes and into the cytosol is not new (see for references on this subject the recent review of Sandvig and van Deurs [13]). DT, after binding to its receptor membrane-bound precursor HB-EGF, is internalized by a process called receptor-mediated endocytosis. Receptor-mediated endo-/transcytosis is a well-known safe and effective cargo-carrying transport mechanism for the selective targeting of drugs to the brain [2]. However, the use of specific ligands for DTR to carry drugs directly across the BBB into the CNS by a mechanism involving receptor-mediated endo-/transcytosis, as has been described for, e.g., the transferrin receptor, has never been appreciated earlier. In fact, only the non-toxic C fragment of the tetanus toxin protein (TTC or Tet451) and the non-toxic derivative of the tetanus toxin protein (Glu234 substitution by Ala) have been exploited to carry drugs into the CNS, however, by a clearly distinctive mechanism of action (as compared to receptor-mediated endo-/transcytosis at the BBB), involving uptake into peripheral nerve endings followed by retrograde axonal transport to their cell body and trans-synaptic transfer to central neurons [14]. Even though constitutive and disease-induced HB-EGF expression in neurons, glial cells, and blood vessels in the brain of rats was already described earlier by Mishima et al. [7], Nakagawa et al. [8], Hayase et al. [9], and Tanaka et al. [10], none of these authors appreciated the opportunity for the targeted delivery of drugs coupled to ligands of the DTR to (intracellular compartments in) these cells in the brain. This oversight is best explained by the fact that rodent HB-EGF is not a receptor for DT. In fact, in rodents, the permeability of DT across the microvasculature of brain tumors is equal to or less than the permeability of other large proteins [15], even though it could effectively kill tumor cells within the brain after passive diffusion [16]. Therefore these studies only reported on the auto-and juxtacrine growth-and adhesion factor properties of HB-EGF.

DTR-targeted drug delivery to the brain is likely to outperform competing brain drug delivery systems since these may involve potential safety hazards. Other endogenous internalizing receptors, such as the insulin-, transferrin, or LDL-related protein 1 (LRP1) receptor, may result in the obstruction of brain transport of essential compounds (like insulin or iron), or result in dangerous interactions with endogenous substrates as was published for melanotransferrin/p97 which activates plasminogen [17] and co-localizes with beta-amyloid [18]. The DTR has no endogenous ligands and thus neither competition from endogenous ligands nor blockade of transport to the brain of essential nutrients is to

be expected. Increased adsorptive-mediated endocytosis, the mechanism of action used by peptide vectors, may result in neurotoxic side effects as this goes against the neuroprotective nature of the BBB. Usually, general drug delivery technologies only alter the distribution or the kinetics of the drug throughout the whole body, which then merely results in a moderately larger brain uptake. This increases the chance of peripheral side effects. All in all, the DTR seems to be a human applicable, safe, and effective uptake receptor for the targeting of drugs to the brain.

### Acknowledgements

The authors thank Heleen Voorwinden for her technical support on the cell culture, Johan Pragt and Fred Kokkedee for their support in the animal facilities, Alfred Nijkerk and Corine Visser for helpful discussions on drug targeting to the brain. This work was financially supported by grant 014-80-119 from STIGON (“Stimuleringsprogramma Innovatief Geneesmiddelonderzoek en Ondernemerschap in Nederland”, ZonMw, The Hague, The Netherlands).

### References

- [1] A.G. de Boer, I.C. van der Sant, P.J. Gaillard, The role of drug transporters at the blood–brain barrier, *Annu. Rev. Pharmacol. Toxicol.* 43 (2003) 629–656.
- [2] W.M. Pardridge, Drug and gene targeting to the brain with molecular Trojan horses, *Nat. Rev., Drug Discov.* 1 (2) (2002) 131–139.
- [3] M. Demeule, et al., High transcytosis of melanotransferrin (P97) across the blood–brain barrier, *J. Neurochem.* 83 (4) (2002) 924–933.
- [4] M.B. Rennels, et al., Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 in United States infants, *Pediatrics* 101 (4 Pt. 1) (1998) 604–611.
- [5] S. Buzzi, et al., CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients, *Cancer Immunol. Immunother.* 53 (11) (2004 Nov.) 1041–1048.
- [6] G. Raab, M. Klagsbrun, Heparin-binding EGF-like growth factor, *Biochim. Biophys. Acta* 1333 (3) (1997) F179–F199.
- [7] K. Mishima, et al., Regional distribution of heparin-binding epidermal growth factor-like growth factor mRNA and protein in adult rat forebrain, *Neurosci. Lett.* 213 (3) (1996) 153–156.
- [8] T. Nakagawa, et al., Neuronal and glial expression of heparin-binding EGF-like growth factor in central nervous system of prenatal and early-postnatal rat, *Brain Res. Dev. Brain Res.* 108 (1–2) (1998) 263–272.
- [9] Y. Hayase, et al., Expression of heparin-binding epidermal growth factor-like growth factor in rat brain, *Brain Res.* 784 (1–2) (1998) 163–178.
- [10] N. Tanaka, et al., Heparin-binding epidermal growth factor-like growth factor mRNA expression in neonatal rat brain with hypoxic/ischemic injury, *Brain Res.* 827 (1–2) (1999) 130–138.
- [11] C.C. Visser, et al., Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells in vitro, *J. Drug Target.* 12 (3) (2004) 145–150.
- [12] P.J. Gaillard, et al., Establishment and functional characterization of an in vitro model of the blood–brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes, *Eur. J. Pharm. Sci.* 12 (3) (2001) 215–222.
- [13] K. Sandvig, B. van Deurs, Membrane traffic exploited by protein toxins, *Annu. Rev. Cell Dev. Biol.* 18 (2002) 1–24.
- [14] Y. Li, et al., Recombinant forms of tetanus toxin engineered for examining and exploiting neuronal trafficking pathways, *J. Biol. Chem.* 276 (33) (2001) 31394–31401.
- [15] C.J. Wrobel, et al., Diphtheria toxin effects on brain-tumor xenografts. Implications for protein-based brain-tumor chemotherapy, *J. Neurosurg.* 72 (6) (1990) 946–950.

- [16] F. Arguello, et al., Two serologic markers to monitor the engraftment, growth, and treatment response of human leukemias in severe combined immunodeficient mice, *Blood* 87 (10) (1996) 4325–4332.
- [17] M. Demeule, et al., Regulation of plasminogen activation: a role for melanotransferrin (p97) in cell migration, *Blood* 102 (5) (2003) 1723–1731.
- [18] W.A. Jeffries, et al., Reactive microglia specifically associated with amyloid plaques in Alzheimer's disease brain tissue express melanotransferrin, *Brain Res.* 712 (1) (1996) 122–126.

## Discussion

*Gabathuler:*

I've one question about your in vitro model. When you look at trancytosis you look at something, it has crossed astrocytes, so it's not picked up by astrocytes?

*Gaillard*

This receptor is not recognized in rodent cells, like in the rat astrocytes, and we were perhaps a little bit lucky with that in this particular experiment.

*Sugiyama*

Approximately 10 years ago I tried to use the poliovirus receptor for targeting to the brain, because the poliovirus is known to infect the brain fairly specifically. We did quite a lot of studies and, of course, we found the receptor for poliovirus on the blood–brain barrier. However, finally, we quitted because the internalization or trancytosis efficacy is not high enough for the drug delivery purpose. It seems to me, from my experience, that whether you can use this system for delivery purposes depends on many factors. One is the density of the receptor on the brain endothelial cells and, of course, the affinity, the internalization rate constant and the recycling rate constant, as well as the trancytosis capacity or rate. Do you have any idea about receptor density, the internalization turnover, or half-life? If you use your in vitro system I think that it is easy for you to determine the efficacy of trancytosis by using the so-called pulse-chase method. You can use the labelled compound and then you can follow the ligand together with the receptor, by the internalization. After that, you can wash the vessel in each side. Then you measure how much the ligand comes out onto this side. By doing so, you can know the efficacy of the trancytosis.

*Gaillard*

I think that in the trancytosis experiment, we did more or less that, because we added the compound to the apical compartment and transferred the well at every time point to a clean dish to get the release on the basolateral side. It's already one of the ways we do it.

*Gabathuler*

What we did was basically load at 4°, look at the binding, wash and then heat up at 37°, and look at how efficiently it's transported. In our case, it's transported very efficiently. In human capillaries, when you look at the binding at 4° and then you look at saponin treatment, you have 80 to 90% of the receptor inside the cell, so you have an internal compartment of receptors that is available, and a very fast recycling on this type of receptor. When you look at affinities of these receptors for the ligands, the affinities are on the level of 0.1 µmol, 100 nmol, 10 nmol, which is, when you look at antibodies a much lower affinity, the way you compensate that is because you have lots of receptors on the blood–brain barrier. So the system is efficient to trancytose something to the brain, even when it has low affinities.

*Aerts*

I would like to make a comment that may add to the discussion on trancytosis versus endocytosis. I'm not an expert on brain endothelial cells but I know something, for example, about lysosomes in macrophages, and what has become clear in that field in the past 5 years is that there's actually a relevant process of lysosome extrusion, so lysosomes are no longer considered in many cell types as the dead end. Cells can get rid of lysosomes by simply fusing them with the plasma membrane, so, in principle, it seems to me possible that also the endothelial cells in the brain could carry out such a process. And if the extrusion takes place at the other side, obviously there will be a trancytotic phenomenon, so maybe the discrimination between trancytosis and endocytosis could be a little bit arbitrary if the final result is just the passage of the barrier.

*Langedijk*

Will you not, after long therapy, generate more and more neutralising antibodies, that may cause some kind of problem?

*Gaillard*

That's always a risk when you are using exogenous proteins, especially bacterial proteins. On the other hand, the administration of bacterial proteins by intravenous infusion is not the best way to get an immune response. Normally, that more or less excludes an immune response against the protein, you need other sites in the body to elicit that, but obviously in the long run, you will end up with problems.

*Galla*

In your case, do you really need the astrocyte co-culture, because if you would like to quantify the trancytosis, even if there is no receptor on the astrocytes, they, nevertheless, occupy the surface. That means that you have a lot of problems to say how many molecules are transported per area, because you never know what could come out on the other side. If you're working on a filter and you know the filter pores you can quantify. With those big molecules, wouldn't a simple endothelial culture be good enough for your system?

*Gaillard*

I think you're right that it is good enough, but I have another point of view on this. I think it's very good to have the astrocytes in, because, *in vivo*, they are there as well and, I think, why not measure it closely mimicking the *in vivo* situation?

*Galla*

Although knowing that the astrocytes are important, is it necessary in an *in vitro* system to make the system more complicated than it has to be?

*Gaillard*

If we go back to how we found the receptor, we only found the receptor because we were using the astrocytes. You do not see the disease induced up-regulation of the receptor without the astrocytes. These astrocytes are not there just by accident.

*Galla*

What was the effect of the MMPs? Do these prevent the cleavage of the conjugate?

*Gaillard*

They actually cleave the membrane-bound receptor, making it the soluble HBEGF. That's the normal physiology of the receptor, to be cleaved and to go to the EGF receptor, and that process can be blocked with proteases, and then you

still have the membrane-bound receptor available for targeting. That's the basic idea.

*Abbott*

I agree with you about the presence of the astrocytes. I think that, from what we know, the polarity of the endothelial cells may be critically dependent on their presence on the abluminal side, because they help to tell the cell which is apical and which is basal, and for these critical transcytosis mechanisms I think that may be important. Also, you mentioned some future animal studies... but clearly these won't be rat or mouse. Are guinea pig or gerbil or other rodents suitable?

*Gaillard*

With gerbil, we don't know. I asked one of the big experts in the diphtheria toxin field, and he didn't know. So then I stopped there. The studies will be in hamsters. We are now setting up a stroke model in hamsters together with an institute in the Netherlands, and guinea pig is also OK.

*Abbott*

But might it just make it harder to do transgenic studies?

*Gaillard*

Well, there is a combination of a group in Dallas and a Korean group that just generated a transgenic mouse with the human diphtheria toxin receptor. It's available and they agreed that we could use it for this purpose. We do not have it yet in the lab. We are hoping that we can use that mouse, of course, for further studies that would make life a lot easier.

*Stanimirovic*

I'm interested in how your system might work in terms of brain selectivity? In other words, are brain endothelial cells particularly enriched with the receptor, compared to peripheral endothelium? If you are to achieve selectivity in brain delivery, would the system lend itself to that?

*Gaillard*

In non-disease conditions, from what I've seen now from literature data, the expression level on endothelial cells is the same throughout the body, but if you have a disease condition in the brain, you might get a stronger expression there and you might have some better penetration in the brain. That still needs to be proven, to see if that's really an effective way, of course. But that might increase the disease targeting.

*Scherrmann*

I have a small question on the kinetics of your uptake. It seems that you start to see your compound after 2 h. Is it correct?

*Gaillard*

Yes, that's correct. That could also be the limit of detection of the assay we use.

*Scherrmann*

The question is important when you compare your uptake kinetics with that of the cell-penetrating peptides, because the process takes a few seconds. It could mean that the uptake mechanisms are different.

*Smith*

To what extent could this technology be used to take things out of brain? Is this a way of getting rid of some things that accumulate in neurodegenerative processes? Maybe it

would be possible to develop a derivative that would go in bind to toxic peptides or factors and help in a sense to pull them out?

*Gaillard*

That's indeed a very interesting suggestion. We know that the receptor is also present on the basolateral side, and up till now I felt that it was a disadvantage, but maybe we can turn that around and see it as an advantage as well.

*Van Tellingen*

So there is not an animal model available yet. Does it mean that you don't have any validation data yet in any animal model, or did you do something in guinea pigs?

*Gaillard*

We did distribution studies in guinea pigs, but for disease models, we are still working on that. So we do not have any disease-modifying validation of this. Our strategy was not to set up ourselves a lot of different disease models, but just to go to companies that have a drug and that have a real need there. We think that that could speed up the development of this technology a lot more, rather than trying to do that ourselves.

*Sugiyama*

Sorry to make such a disappointing comment but I must refer to our experience with poliovirus. Actually, the polio virus is known to infect, known to cause toxicity on the brain, and we actually did use the human receptor in transgenic mice. The receptor really does exist in various kinds of tissues, not only in the brain but also in the liver, kidneys, the spleen, many other kinds of tissues, but polio virus shows a very rapid growth just in the brain and that is the reason why toxicity is caused by polio virus only in the brain, even when the targeted amount of polio virus is very low.

*Gaillard*

We are working with a non-toxic bacterial protein. It's a protein, not a growing bacterium, so we don't expect any problems, and the protein has been given to humans.