Peptide Derived from HIV-1 TAT Protein Destabilizes a Monolayer of Endothelial Cells in an in Vitro Model of the Blood-Brain Barrier and Allows Permeation of High Molecular Weight Proteins*

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Background: An in vitro model for studying BBB opening and entry of impermeable substances was constructed.

Results: A brain capillary endothelial cell monolayer was disrupted and opened to the penetration of impermeable therapeutic agents by C-TAT peptides.

Conclusion: Experimental conditions enabling the blood to brain entry of impermeant therapeutic agents were established.

Significance: Approaches toward overcoming states of major brain disorders were initiated.

Most chemotherapeutic agents are blood-brain barrier (BBB) impermeants. HIV-1-derived TAT protein variants contain a transmembrane domain, which may enable them to cross the BBB and reach the brain. Here we synthesized CAYGRK-KRRQRRR, a peptide containing a cysteine moiety attached to the N terminus of the transmembrane domain (C-TAT peptide), and studied its effects in an in vitro BBB model, which we found to reflect penetration by a receptor-independent pathway. Incubation of the brain capillary endothelial cell monolayer with 0.3–0.6 μmol/ml of this C-TAT peptide, for a period of 1–2 h, destabilizes brain capillary endothelial cell monolayer and introduces the ability of impermeant therapeutic agents including high molecular weight proteins to penetrate it substantially. The cysteinyl moiety at position 1 of the C-TAT peptide contributes largely to the destabilizing potency and the penetration efficacy of impermeant substances. The destabilizing effect was reversed using heparin. In summary, experimental conditions allowing a significant increase in entry of impermeant low and high molecular weight substances from the luminal (blood) to the abluminal side (brain) were found in an in vitro BBB model reflecting in vivo protein penetrability by a receptor-independent pathway.

A large variety of therapeutic agents with potential for treating brain disorders are not in clinical use because they are unable to cross the blood-brain barrier (BBB) following peripheral administration (1). Even small bioactive peptides penetrate the BBB only if highly lipophilic (2). Furthermore, therapeutically relevant proteins such as herceptin (trastuzumab, a humanized IgG1 molecule) become ineffective, when HER-2-positive metastases have reached the brain (3).

The BBB is formed in part by tight junctions between adjacent endothelial cells that make up the cerebral capillaries. Tight junction proteins connect neighboring endothelial cells to each other in a noncovalent fashion (4). Several pathological conditions are known to result in marked disruption of the tight junctions of the BBB (5–8). Such disruptions can lead to undesirable pathological consequences but might permit the penetration of therapeutic agents relevant for brain disorders. Clinically, all attempts so far to enhance penetration of therapeutic proteins via the BBB in quantities sufficient to treat major brain disorders were unsuccessful (9).

A truncated form (71 amino acids) of the HIV-1 TAT (transactivator of transcription) protein was found to have the unique ability to enter cells through the plasma membrane and accumulate intracellularly (10, 11). This is believed to take place through a plasma membrane bilayer component, in a receptor- and transporter-independent fashion (12). This feature was attributed to a nine-amino acid stretch of basic amino acids of sequence RKRRQRRRR, located in region IV of TAT protein (13). Peptide fragments containing this protein transduction domain preserve this feature as well (14, 15). Studies in rodents in vivo revealed that both TAT protein variants and peptides or fragments containing this protein transduction domain are capable of penetrating the BBB and accumulate in brain tissue (16). This was the rationale to covalently link the peptide to biologically active proteins for delivering them either into peripheral tissues or to the CNS (13–15). Indeed such peptide-

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4 The abbreviations used are: BBB, blood-brain barrier; ES, electrospray; HSA, human serum albumin; TEER, transependothelial electrical resistance; Pe, permeability; PBEC, porcine brain endothelial cell; MAL, maleimide; PBEC-M, PBEC monolayer.
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proteins from the periphery to the brain are still needed. In this study, we have synthesized cysteine-containing 13-amino acid fragments, based on the protein transduction domain of the TAT protein, postulating that the efficacy of this protein to cross the plasma membrane and to disrupt BBB tightness are interrelated. Efforts were then undertaken to modify this peptide to a form that preserves its BBB disrupting property but with reduced penetrability into cell interiors. We have used an in vitro experimental system composed of tight junction-linked primary porcine brain endothelial cells (PBEC) grown in culture (20, 21). This in vitro experimental system reflects well BBB in vivo with regard to permeability (Pe) in agreement with findings made in rodents in vivo (22). The tightness of this monolayer is determined by measuring the transendothelial electrical resistance (TEER). As relevant for Pe, this parameter is recognized as one of the most accurate and sensitive measures of BBB integrity (23). As for the BBB in mammals, major tight and adherence junction proteins are expressed when endothelial cells contact each other (20, 21). This experimental system allowed us to determine the disruption efficacy and the permeation potency of a large variety of therapeutic agents, including high molecular weight proteins. Our efforts in this direction are described in this report.

EXPERIMENTAL PROCEDURES

Materials

C-TAT peptide (cysteine- and TAT-containing peptide, CAAGRKKKRRQRRR) was synthesized by the manual conventional synthesis. An Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy was employed throughout the peptide chain assembly. The calculated mass of the C-TAT peptide is 1734.07 Da, found by electrospray single quadrupole mass spectroscopy analysis: ES (electrospray) = 1733.53 ± 0.04 Da. HSA, 5,5'-dithio-bis (2-nitrobenzoic acid), N-ethylmaleimide, and fluoresceine isothiocyanate were purchased from Sigma. PEG5-MAL (a 5-kDa product thus obtained was dialyzed for 10 h against H2O and lyophilized). The reaction was continued for 5 min at 25 °C. The methylformamide was then added (2 molar excess over the peptide) and the calculated mass for C-TAT-MAL is 1859 Da. Found by electrospray single quadrupole mass spectroscopy analysis: ES = 1858.49 ± 0.21.

H2O5-S-C-TAT (a C-TAT peptide in which the cysteine moiety was converted to cysteic acid) was prepared by performic acid oxidation of the peptide. C-TAT peptide (12.2 mg, 7 μmol) was dissolved in 0.45 ml of formic acid. Hydrogen peroxide (50 μl) was then added, and the reaction was carried out at 25 °C over a period of 2 h. Performic acid was then removed by evaporation. The product obtained was dissolved in H2O and lyophilized. This procedure was repeated three times. H2O5-S-C-TAT peptide contains cysteic acid moiety (mol/mole) as verified by amino acid analysis following acid hydrolysis. The calculated mass is 1782 Da, found by electrospray single quadrupole mass spectroscopy analysis: ES = 1781.52 ± 0.06 Da.

Acetylated C-TAT peptide was prepared by dissolving 12.2 mg of C-TAT in a mixture of 1:1:0.5 of H2O, pyridine and acetic anhydride. The reaction was carried out at 0 °C. The medium was evaporated, and the product was dissolved, obtained in H2O, and lyophilized. This procedure was repeated three times. Acetylated C-TAT peptide was then dissolved in 0.1 M Na2CO3 (pH 10.3, 1 h, 25 °C) for deacetylating the tyrosyl and the cysteinyl moieties of this derivative. The pH was then dropped to 6.0 with HCl, and the product was frozen until used.

Fluoresceine-labeled HSA was prepared by dissolving 67 mg of HSA (1 μmol) in 0.5 ml of 0.1 M Na2CO3 (pH 10.3). Fluoresceine isothiocyanate 1.4 mg (3.6 molar excess over HSA) was then added, and the reaction was carried out for 1 h at 25 °C. The reaction mixture was then loaded on a Sephadex G-50 column (1.7 × 14 cm), equilibrated, and run in the same buffer. The tubes containing fluoresceine-labeled HSA were pooled, dialyzed against H2O, and lyophilized. Flures-HSA (HSA derivative containing 1.7 mol of fluoresceine/mol of HSA) prepared by this procedure contains 1.7 ± 0.2 mol of fluoresceine/mol of HSA as determined by its absorbance at 500 nm using ε500 = 62,000.

Cationized Flures-HSA was prepared by dissolving 20 mg of Flures-HSA in 1.0 ml of 1 M 1,3-diaminopropane or in 1 ml of 1 M arginine-amide. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (70 mg) was then added. The pH was adjusted to 6.0 ± 0.1. The reaction was carried out for 2 h. The derivatives thus obtained were dialyzed against H2O for 2 days with several changes of H2O and lyophilized. Flures-HSA that was cationized with arginine-amide contains additional 64 ± 3 moieties of arginine-amide/mol of Flures-HSA as determined by amino acid analysis following acid hydrolysis. The protein concentration was calculated according to alanine (62 residues) and glycine (12 residues).

Biological and Chemophysical Procedures

Radiolabeling with Na125I—Radiolabeling of peptides and proteins with Na125I was carried out essentially by the procedure of Hunter and Greenwood (24) with slight modifications that were described in detail in Ref. 25.

Primary Cultures of Brain Endothelial Cells—Primary cultures of brain endothelial cells were isolated from freshly collected porcine brains as described previously (21, 26). These PBEC were validated as true BBB endothelial cells in previous
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studies (21, 26). The purity of the culture was confirmed by specific staining for von Willebrand factor (20).

In Vitro BBB Models and Transendothelial Electrical Resistance Measurements—In a typical experiment, PBEC were seeded at a density of 100,000 PBEC/cm² on a microporous membrane of a Transwell insert (Corning Costar, Acton, MA) placed into a 12-well plates. The cells were cultured in plating medium for up to 3 days until they reached confluency. The medium was replaced with a serum-free medium (assay medium) for an additional 24–48 h, and the integrity of this cellular barrier was determined by measuring TEER. A decrease in TEER reflects an increase in permeability and a loss of barrier function. TEER of the filter insert was recorded using an Endohm chamber connected to an EVOM resistance meter (World Precision Inst., Inc., Sarasota, FL). The TEER of each filter insert was calculated by subtracting the TEER of the microporous membrane without PBEC and is reported as Ωcm². When using a co-culture BBB in vitro system, Transwell inserts seeded with glial cells at the bottom were used as blinks. The co-culture system was used in a “contact” configuration as described in detail in Ref. 20. For testing the effects of the different compounds on TEER, they were diluted in assay medium at the desired concentrations and added to the luminal (to mimic blood to brain passage) or abluminal (to mimic brain to blood passage) side of the inserts.

Media—Plating medium is composed of newborn calf serum (10%), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and gentamicin (0.1 mg/ml), all dissolved in Earl’s medium 199 (Sigma). The assay medium consists of L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), gentamicin (0.1 mg/ml), and hydrocortisone (550 nM) in DMEM diluted 1:1 in Ham’s F-12 medium (Biological Industries, Beit Haemek, Israel).

Permeability Measurements—The PBEC permeability of this in vitro BBB model to radiolabeled compounds ([14C]sucrose, [125I]HSA, and [125I]Herceptin) and fluorescently labeled compounds (doxorubicin or Flures-HSA) was carried out as described in detail in Refs. 20 and 27 with slight modifications. The tested compounds were added at the luminal side of the Transwell inserts. During the transport assay, the cells were incubated at 37 °C. Samples (500 µl) were collected every 10 min over a period of 40 min from the abluminal side and measured for their 14C or 125I content or for their fluorescent intensity. Pe values of filters having no PBEC seeded on them were subtracted. Pe was obtained from the slope of the calculated clearance curve as described in Ref. 27.

RESULTS

Cationized Albumin Permeates through the PBEC Monolayer—We prepared Flures-HSA and further cationized it by linking either 1,3-diaminopropane or arginine-amide, converting a major part of the carboxylate moieties (~60%) into positively charged residues (“Experimental Procedures”). Both versions of fluorescently labeled cationized HSA penetrated this PBEC monolayer efficiently (14–17 cm/s × 10⁻⁶; Fig. 1A). Both derivatives had a marked effect in reducing TEER (Fig. 1B), suggesting that penetrability is secondary to destabilizing the tightness of this monolayer. Noncationized HSA neither penetrated nor destabilized this PBEC monolayer (Fig. 1). Thus, this in vitro experimental system resembles BBB penetrability in vivo, with regard to proteins that can enter this barrier by absorptive mediated transendoctyosis (1, 28).

Synthesizing and Characterizing a Peptide That Opens BBB, Based on TAT Protein Variants—Several TAT-containing peptide versions were synthesized: of these, we have selected as the first prototype a version containing a single cysteinyl moiety at position 1 combined with fragment 46–57 of TAT protein, which contains the transduction domain (13). Table 1 summarizes several of the properties of this C-TAT peptide (CAY-GRKKRRQRRR). It migrates as a single peak on HPLC column with a retention time value of 4.605 min. It absorbs at 278 nm with extinction coefficient (ε₂₇₈) of 2400 ± 50, a value that is 1.71 times higher than that expected from a peptide containing a single tyrosyl moiety (ε₂₇₈ = 1400). Mass spectrum analysis revealed the corrected mass as calculated (1734.07 Da). Performic acid oxidation of this peptide yielded 1.0 ± 0.1 mol/mol cysteic acid, as determined by amino acid analysis following
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**TABLE 1** Chemical features of C-TAT peptide

| Characteristic | Value |
|----------------|-------|
| Migration on analytical HPLC retention time | Single peak, 4.605 min |
| Absorbance at 278 nm | ε₂₇₈ = 2400 ± 50 |
| Mass spectra | Calculated 1734.07 daltons, found (ES) 1733.53 ± 0.04 daltons |
| Cysteic acid content following performic acid oxidation | 1.0 ± 0.1 mol/mol |
| Solubility in H₂O | > 20 mg/ml |

- Conducted with a linear gradient from 0 to 100% of solution A (0.1% TFA) to solution B (acetonitrile-H₂O 75:25 in 0.1% TFA) using a chromolith Rp 18e (100 x 4 mm) column.
- Determined by UV spectroscopy. Peptide concentration was determined by acid hydrolysis of 20-μl aliquot followed by amino acid analysis and calculated according to alanine and glycine (one residue each).
- Mass spectroscopy was determined by the electrospay ionization technique.
- Cysteic acid content was determined by acid hydrolysis and amino acid analysis of an aliquot following performic acid oxidation ("Experimental Procedures"). Peptide concentration was calculated according to alanine and glycine (one residue of each).

Acid hydrolysis. C-TAT peptide is highly soluble in H₂O (>20 mg/ml).

**C-TAT Peptide Decreases TEER in a Concentration- and Time-dependent Fashion**—Fig. 2A shows the effect of several concentrations of C-TAT peptide placed at the Transwell luminal side, which resembles the blood side of the BBB, caused by the asymmetry alignment of the cells grown on collagen-coated inserts (26), on TEER over a period of 2 h. At a C-TAT peptide concentration of 0.6 μmol/ml, TEER values were decreased in a time-dependent fashion amounted to 47, 13, and 2% of initial value following 0.5, 1.0, and 2 h of incubation, respectively. At 0.3 μmol/ml C-TAT peptide, TEER decreased to 48, 27, and 16% of initial value following 0.5, 1.0, and 2 h of incubation, respectively. A lower concentration of the C-TAT peptide (0.06 μmol/ml) decreased TEER by 26%, following 1 h of incubation with no further decrease at 2 h (Fig. 2A).

We also studied the destabilizing effect of the C-TAT peptide placed at the abluminal side (“brain” side). TEER value has decreased as well with time, although at a lower rate, as compared with the same peptide concentration placed at the luminal side. However, in both cases TEER values decreased to ~2–7% of initial value following 2 h of incubation.

Lastly, we have compared the destabilizing effect of the C-TAT peptide, side by side on a monoculture of PBEC as described above and a contact co-culture of endothelial cells on one side of the insert and glial cells seeded on the bottom of the Transwell inserts (20). TEER was ~1.5 times higher in the co-culture (707 ± 16 versus 458 ± 30 Ω·cm², respectively). C-TAT peptide, however, was nearly equipotent in decreasing TEER with time (τ₁/₂ = 0.4 ± 0.02 h; Fig. 2B). Thus, the added glial cells enhance significantly BBB tightness but not the efficacy of the C-TAT peptide to disrupt it, suggesting that the effect of C-TAT on the barrier properties is primarily on the endothelial cells.

**Two Patterns of BBB Opening by C-TAT Peptide**—Heparin was documented to associate with TAT protein transduction domain (29). Fig. 3 shows that heparin associates with the C-TAT peptide as well. Heparin, at a 2:1 molar ratio suppresses the peptide efficacy to reduce TEER over a period of 2 h (Fig. 3). We therefore added heparin at 0, 1, 5, and 30 min following the addition of the C-TAT peptide to the PBEC monolayer (PBEC-M), and TEER values were monitored over a period of 2 h. Exposure of PBEC-M to the C-TAT peptide for a period of 1 min prior to neutralizing it with heparin reduced TEER by ~60%, and this was followed by nearly full recovery of TEER within a period of 2 h. Nearly the same pattern is seen following 5 min of exposure of PBEC-M to the peptide prior to neutralizing it. More prolonged exposure (30 min) of PBEC-M to the peptide prior to neutralizing it, yielded large decrease in TEER with little restoring efficacy in the 2-h time frame shown in Fig. 3, although after 24 h, PBEC-M has restored to large extent its TEER values (data not shown). Thus, C-TAT peptide appears to yield two distinct patterns of BBB opening. The first one takes place within a short (1 min) transitory exposure and is nearly fully reversible, where the second pattern takes place...
following prolonged (30 min) exposure of the PBEC-M to the peptide, yielding a more extensive and less reversible BBB opening pattern.

Cysteine 1 of the C-TAT Peptide Contributes Significantly to the PBEC-M Destabilizing Potency of the Peptide—Initially we elongated the C-TAT peptide by adding additional arginine moiety at the C-terminal position. This 14-amino acid peptide (CAYGRKKRRQRRRR) was as effective as the shorter C-TAT version (not shown), indicating that additional positive charge at the C-terminal end is not required for enhancing its destabilizing efficacy. Subsequently we prepared two additional versions of this longer C-TAT peptide. The first one lacks its cysteinyl moiety at position 1 (AYGRKKRRQRRRR; TAT peptide), and in the second version the cysteinyl moiety was replaced with valine (VAYGRKKRRQRRRR; V-TAT peptide). Valine has rather similar atomic volume and degree of hydrophobicity as cysteine but lacks the weakly acidic and the reducing efficacy of cysteine. As shown in Fig. 4, the C-TAT peptide is approximately twice as potent as the TAT peptide in disrupting PBEC-M, as well as capable of fully reducing TEER, 2 h after addition. V-TAT peptide was nearly as potent as the C-TAT peptide in this respect (Fig. 4). Thus, the destabilizing efficacy of TAT containing peptides is significantly enhanced if either cysteine or valine are present at the N-terminal end. It appears that a hydrophobic side chain moiety, e.g., -CH2-SH (cysteine) or -CH(CH3)2 (valine), is responsible for this increase in potency. Further derivatization of the sulphydryl moiety of the C-TAT peptide yielded inactive derivatives.

**Structure-Function Relationships**—Table 2 shows the potencies of several C-TAT peptide derivatives to reduce TEER value of PBEC monolayer following 1 h of incubation with 0.6 μmol/ml of each derivative. Derivatization of the free cysteinyl moiety with NEM, linking to it PEG5-MAL, or oxidizing it to cysteic acid, yielded derivatives having reduced BBB disrupting potency. Interestingly, acetylation of the C-TAT peptide under conditions that preserve the cysteinyl-moiety in its reduced form yielded a C-TAT peptide derivative that preserved ~47% of its destabilizing potency (Table 2). Thus, the positive charges of the two lysine moieties in this peptide can be replaced by noncharged amino acid moieties.

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![Figure 3](image3.png)

**Figure 3.** PBEC monolayer recovers after neutralization of C-TAT with heparin. C-TAT was added to the luminal side of the in vitro BBB model at 0.6 mM, and heparin was added at 2:1 molar ratio in several time points afterward. TEER values were then monitored. The data are expressed as percentages of initial TEER values normalized to control inserts and presented as the means ± S.E. values (n = 3 inserts).

![Figure 4](image4.png)

**Figure 4.** Cysteine 1 of the C-TAT peptide contributes significantly to the peptide PBEC-M destabilizing potency. Two versions of the longer C-TAT peptide were synthesized. The first one lacks its cysteinyl moiety at position 1 (AYGRKKRRQRRRR; TAT), and in the second version the cysteinyl moiety was replaced with valine (VAYGRKKRRQRRRR; V-TAT). The peptides were added to the luminal side of the Transwells with PBEC-cultured in monolayers, and TEER values were monitored over a period of 2 h. The data are presented as the means ± S.E. (n = 3 inserts).

**Table 2**

| Derivative designation | Abbreviated structure | TEER (Ωcm²) | % Destabilization |
|------------------------|-----------------------|-------------|-------------------|
| Control                | —                     | 400±30      | 0                 |
| C-TAT-peptide          | -SH                  | 36±1.4      | 91±4              |
| C-TAT-MAL              | -SO₂H                | 340±7       | 15±2              |
| PEG-MAL-C-TAT          | -SO₂H                | 352±4       | 12±1              |
| HO₂S-C-TAT             | -SO₂H                | 272±6       | 32±2              |
| Acetylated-C-TAT       | -NCO₂H               | 212±4       | 47±2              |

![Image5.png](image5.png)
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**FIGURE 5. Effect of C-TAT peptide treatment on penetration of low molecular weight substances.** PBEC-M in Transwells were incubated with medium alone or with medium containing C-TAT peptide (0.6 µmol/ml) for 2 h at 37 °C. [14C]Sucrose or doxorubicin HCl were then added at the luminal side, and their permeability across PBEC-M to the abluminal side was determined by measuring Pe values every 10 min over a period of 40 min. The results are expressed as the means ± S.E. of the independent assays (n = 3–4 inserts). *, p < 0.05; ***, p < 0.001 versus untreated.

**FIGURE 6. Effect of C-TAT peptide treatment on penetration of high molecular weight substances.** PBEC-M in Transwells were incubated with medium alone or with medium containing C-TAT peptide (0.6 µmol/ml) for 2 h at 37 °C. [125I]HSA or [125I]Herceptin was then added at the luminal side, and their permeability across PBEC-M to the abluminal side was determined by measuring Pe values every 10 min over a period of 40 min. The results are expressed as the means ± S.E. of the independent assays (n = 4–6 inserts). ***, p < 0.01; ***, p < 0.001 versus untreated.

**TABLE 1**

| Pe (cm/sec) × 10^(-6) | C-TAT-peptide treated, PBEC-Monolayer | Non treated, PBEC-Monolayer |
|-----------------------|--------------------------------------|-----------------------------|
| 14C-Sucrose            | 18                                   | 6                           |
| Doxorubicin            | 3                                    | 1                           |

**DISCUSSION**

In this study we were interested in finding conditions that permit significant permeations of impermeant substances including therapeutic proteins from the periphery to the brain. Major attempts have been undertaken worldwide in this direction. A common approach is to link or fuse BBB-impermeable peptide or protein to a “shuttle protein” that enters the BBB by receptor-mediated transendocytosis (30). Such approaches may deliver low (pmol) amounts of a peptide or a protein from the periphery to the CNS. From a clinical standpoint, however, this approach appears inadequate. Other directions were therefore attempted. Brown et al. (31) have demonstrated opening of the BBB in patients with malignant brain tumors using hyperosmolar mannitol. This procedure increased to some extent BBB penetrability of 86Rb⁺ and of [14C]sucrose but had little or no effect in enhancing penetrability of high molecular weight proteins.

In this study we have used an *in vitro* model of the BBB, composed of PBECs grown in culture (20, 21). Initially we have analyzed whether this experimental system reflects BBB penetrability of high molecular weight proteins as well. As validated in rodents *in vivo*, we found that HSA (molecular mass = 66 kDa), an impermeant molecule, becomes permeable following cationization (Fig. 1). This opened to us the options of finding conditions that weakened BBB tightness and allowed penetration of therapeutic proteins, two parameters that are extremely problematic for evaluation *in vivo* in a quantitative fashion.

Several mechanisms have been proposed by which TAT protein disrupts BBB, including the decrease of tight junction proteins expression and relocalization (32–34). It is reasonable to assume that the C-TAT peptide may have effects similar to those of the TAT protein on the expression of tight junction proteins. This would suggest a paracellular passage after C-TAT peptide treatment. In designing our BBB-disrupting peptide prototype, we hypothesized that in addition to the C-TAT peptide prototype, we hypothesized that in addition to the C-TAT peptide, we compared [125I]Herceptin penetration following disruption with the C-TAT peptide, we compared [125I]Herceptin penetration with both systems under identical experimental conditions. Pe of [125I]Herceptin in the co-culture system amounted to 43 ± 3% of that obtained in the monoculture system. However, the same extent of penetration was reached by increasing the incubation time with the C-TAT peptide to 3 h or doubling its concentration (data not shown).
of the TAT protein may be required to extend the BBB disruption potency of a transduction domain-containing fragment. Postulating that such an additional site must be a conserved entity among TAT protein variants, we have selected a cysteiny l moiety as a representative of the seven very conserved cysteines of region II. NMR studies suggested that the flexibility of the C-TAT protein allows regions II and IV to be in close proximity to each other (35, 36). For that reason we have placed the cysteiny l moiety at position I for allowing its approach to the vicinity of the transduction domain if the appropriate structural alterations do take place at aqueous physiological media. Indeed the inclusion of a cysteiny l moiety at position I yielded a TAT-containing peptide that is considerable more potent in destabilizing the PBEC monolayer (Fig. 4). A free cysteiny l side chain moiety (-CH2-SH) appears to be required. Its derivatization yields less potent derivatives (Table 2). A low concentration of the C-TAT peptide (0.3–0.6 μmol/ml) destabilizes PBEC monolayer within a period of 1.5–2 h (Fig. 2). Short (1 min) exposure of PBEC-M to the peptide followed by its neutralization with heparin yielded 60% decrease in TEER within 30 min followed by nearly full TEER recovery within the next 2 h (Fig. 3). This desirable transitory opening feature is important for clinical implications.

Although not definitive yet, our efforts to obtain a peptide derivative capable of disrupting BBB but lacking its ability to penetrate into cell interiors were not successful so far. For example, PEG5-MAL-C-TAT peptide (a conjugate of PEG5-MAL linked to the cysteine moiety of the C-TAT peptide) is impermeable into cell interiors6 and ineffective as well in destabilizing the PBEC monolayer (Table 2). It therefore seems that these two functions may be interrelated and cannot be dissociated. From a clinical point of view, this implies that in vivo experimental conditions need to be found to bring the C-TAT peptide into close proximity to the BBB, to minimize its uptake by peripheral tissue. Considering the usage of cationized proteins as BBB-opening agents, the efficiency is dependent on the dosage and the type of cationization. Although difficult to compare, we found on a molar basis that 1,3-di-aminopropane-cationized HSA is ~3–5 times more potent than the C-TAT peptide in disrupting and opening PBEC-M to impermeant substances (data not shown).

Finally, we stress that acetylation of the C-TAT peptide under conditions that leave the cysteiny l moiety nonmodified yielded a derivative that preserved ~47% of its BBB-disrupting potency (Table 2). Thus, these two lysyl moieties can be replaced, derivatized, or modified upon developing a second generation of BBB opening peptides with improved therapeutic efficiencies.6

In summary, we have used here an in vitro BBB model that appears to reflect BBB in vivo by at least the following five criteria: very low penetrating capacity of [14C]sucrose; high TEER value (>400 Ω·cm) (20, 21); similar expression pattern of major tight and adherence junction proteins (20); negligible penetration of the C-TAT peptide (0.3–0.6 μmol/ml) destabilizes PBEC monolayer within a period of 1.5–2 h (Fig. 2). Short (1 min) exposure of PBEC-M to the peptide followed by its neutralization with heparin yielded 60% decrease in TEER within 30 min followed by nearly full TEER recovery within the next 2 h (Fig. 3). This desirable transitory opening feature is important for clinical implications.

Although not definitive yet, our efforts to obtain a peptide derivative capable of disrupting BBB but lacking its ability to penetrate into cell interiors were not successful so far. For example, PEG5-MAL-C-TAT peptide (a conjugate of PEG5-MAL linked to the cysteine moiety of the C-TAT peptide) is impermeable into cell interiors6 and ineffective as well in destabilizing the PBEC monolayer (Table 2). It therefore seems that these two functions may be interrelated and cannot be dissociated. From a clinical point of view, this implies that in vivo experimental conditions need to be found to bring the C-TAT peptide into close proximity to the BBB, to minimize its uptake by peripheral tissue. Considering the usage of cationized proteins as BBB-opening agents, the efficiency is dependent on the dosage and the type of cationization. Although difficult to compare, we found on a molar basis that 1,3-di-aminopropane-cationized HSA is ~3–5 times more potent than the C-TAT peptide in disrupting and opening PBEC-M to impermeant substances (data not shown).

Finally, we stress that acetylation of the C-TAT peptide under conditions that leave the cysteiny l moiety nonmodified yielded a derivative that preserved ~47% of its BBB-disrupting potency (Table 2). Thus, these two lysyl moieties can be replaced, derivatized, or modified upon developing a second generation of BBB opening peptides with improved therapeutic efficacies.6

In summary, we have used here an in vitro BBB model that appears to reflect BBB in vivo by at least the following five criteria: very low penetrating capacity of [14C]sucrose; high TEER value (>400 Ω·cm) (20, 21); similar expression pattern of major tight and adherence junction proteins (20); negligible penetration of doxorubicin, [125I]HSA, and [125I]herceptin (Figs. 5 and 6); and the conversion of fluorescein-HSA into a penetrable species following cationization (Fig. 1). This model assisted us in developing, experimental conditions that destabilizes BBB and allows the delivery of therapeutic agents including high molecular weight proteins, from the periphery to the brain in quantities that might be sufficient to treat major CNS disorders and malignant brain tumors.

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