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Increased delivery of TAT across an endothelial monolayer following ischemic injury

Melissa J. Simon^a, Woo Hyeun Kang^a, Shan Gao^b, Scott Banta^b, and Barclay Morrison III^a

^aDepartment of Biomedical Engineering, Columbia University, New York, New York 10027

^bDepartment of Chemical Engineering, Columbia University, New York, New York 10027

Abstract

There is a great need for the development of vehicles capable of delivering therapeutic cargoes across the blood-brain barrier (BBB) and into brain cells. Cell-penetrating peptides (CPPs), such as TAT, present one such solution, and have been used successfully *in vivo* to deliver neuroprotective cargoes to the brain in models of stroke and seizure. However, a significant discrepancy exists in the literature, as other groups have not had the same success. One commonality between the successful studies is a compromised BBB. In this study, we hypothesized that ischemic injury increases the transport of TAT across an endothelial monolayer (comprised of bEnd.3 cells) *in vitro* and, consequently, increases TAT-mediated delivery into astrocytes on the other side. In the 24 hours following *in vitro* ischemia (oxygen-glucose deprivation), transendothelial electrical resistance (TEER) significantly decreased, indicating disruption of BBB integrity. Concomitantly, the transport of a green fluorescent protein (GFP)-TAT fusion protein significantly increased, and the transduction of GFP-TAT into astrocytes cultured on the other side of the endothelial monolayer significantly increased. These results explain why TAT-mediated delivery of therapeutic cargoes is successful in the ischemic brain but not in the uninjured brain with an intact BBB, highlighting the necessity for continued development of delivery vehicles. We conclude that although TAT may not be an efficient vehicle for trans-BBB delivery across an intact BBB, it may have utility in clinical situations when the BBB is disrupted.

Keywords

Cell-penetrating peptide; TAT peptide; endothelial barrier; ischemia

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Barclay Morrison III, PhD, Corresponding Author Department of Biomedical Engineering, 351 Engineering Terrace MC 8904, 1210 Amsterdam Ave, New York, NY 10027, Phone: 212-854-6277, Fax: 212-854-8725, bm2119@columbia.edu.

Melissa J. Simon, MS, Department of Biomedical Engineering, 351 Engineering Terrace MC 8904, 1210 Amsterdam Ave, New York, NY 10027, Phone: 212-854-2823, mjs2150@columbia.edu

Woo Hyeun Kang, MS, Department of Biomedical Engineering, 351 Engineering Terrace MC 8904, 1210 Amsterdam Ave, New York, NY 10027, Phone: 212-854-2823, wk2201@columbia.edu

Shan Gao, PhD, Department of Chemical Engineering, 801 Mudd, 500 W. 120th Street, New York, NY 10027, Fax: 212-854-3054, sg2323@columbia.edu

Scott Banta, PhD, Department of Chemical Engineering, 820 Mudd, 500 W. 120th Street, New York, NY 10027, Phone: 212-854-7531, sbanta@chem.columbia.edu

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Introduction

The cell-penetrating peptide (CPP) TAT, consisting of amino acids 47–57 of the trans-activating transcriptional activator (Tat) from the human immunodeficiency virus 1 (HIV-1), has shown great promise for delivering neuroprotective cargoes, such as Bcl-xL and GDNF, to the brain in *in vivo* models of ischemia [6,16,17,30] and seizure [20]. Delivery of therapeutics to the brain is difficult due to the presence of the blood-brain barrier (BBB). While these results indicate that TAT may be able to overcome that barrier, several other groups have not reported the same success [5,13]. Determining the root cause of this discrepancy is critical for understanding the conditions under which TAT can deliver therapeutics to the brain, and for motivating the continued development of improved CPPs and delivery vehicles. One commonality of the unsuccessful studies was attempted delivery to the healthy brain through an intact BBB.

While many studies have examined the transduction of TAT into cells through the plasma membrane, few have examined the passage of TAT through cell barriers, such as the BBB. We have previously shown that a green fluorescent protein (GFP)-TAT fusion protein was unable to pass through an intact endothelial barrier and enter astrocytes on the other side [27], substantiating the negative *in vivo* results [5,13]. Furthermore, we hypothesized that the positive *in vivo* results may have resulted from the breakdown of the BBB caused by the ischemia or seizure injuries employed in those studies. Both ischemia and seizures cause a breakdown of the BBB [12,15], which may have allowed the TAT construct to enter the brain parenchyma and transduce brain cells.

The goal of our study was to investigate whether ischemic injury increased the permeability of a brain endothelial monolayer thereby leading to a significant increase in the transport of TAT across it. We further hypothesize that this increased transport was sufficient to deliver a protein cargo in measureable amounts to astrocytes on the other side of the model BBB. These results would explain the differences in TAT-mediated delivery observed in the literature and further define the conditions for successful trans-BBB delivery by the TAT peptide and other CPPs. Furthermore, these results would motivate the development of new vehicles with improved capabilities of delivering therapeutics to the brain, which are desperately needed for the treatment of CNS disorders.

Materials and Methods

A total of 60,000 bEnd.3 cells (ATCC, Manassas, VA) were seeded onto the top of poly-L-lysine coated Transwell inserts (1.13 cm² surface area, 0.4 µm pore size, Corning Costar, Lowell, MA) and grown in DMEM supplemented with 10% heat-inactivated newborn calf serum and 4 mM glutamine (Sigma, St. Louis, MO). This immortalized mouse brain endothelial cell line has been characterized extensively as a model of the BBB [4,19,22]. After 4–5 days, the bEnd.3 monolayers were subjected to an ischemic injury by transferring cultures to glucose free medium (DMEM without glucose) pre-equilibrated with 95% N₂ and 5% CO₂. Culture plates were placed in an airtight chamber (Billups-Rothenberg, Del Mar, CA), gassed with 95% N₂ and 5% CO₂ at 15 L/min for 10 minutes and then sealed and incubated for 12 h at 37°C. Control cultures were incubated for 12 h at 37°C in DMEM containing 4.5 mg/mL glucose in 95% air and 5% CO₂.

Astrocyte cultures were obtained from the cortices of 8–10 day old Sprague-Dawley rat pups as described previously [26]. All procedures involving animals were approved by the Columbia University IACUC. Briefly, cortices were digested with papain, triturated, and plated into T75 tissue culture flasks. After an hour, the flasks were vigorously shaken leaving only astrocytes adhered to the flask, resulting in a culture that was over 90%

astrocytes [19]. Astrocytes were cultured for 2 weeks and then plated into 12-well plates at a density of 60,000 cells per well and grown for 3 days in DMEM containing 10% heat-inactivated newborn calf serum and 1 mM glutamine (Sigma).

The GFP and GFP-TAT proteins were made as described previously [14]. Briefly, the GFP gene was encoded in a pRSET-S65T vector [28]. The TAT peptide sequence (YGRKKRRQRRR) was inserted onto the C-terminus of the GFP gene. A 6-histidine tag was present at the N-terminus of the GFP gene, and the proteins were purified using a HisTrap crude FF 5 ml column in an FPLC apparatus (GE Healthcare, Uppsala, Sweden).

Immediately following ischemic injury, the transendothelial electrical resistance (TEER) was measured using an EVOMX Epithelial Voltohmmeter and Endohm-12 chamber electrode (World Precision Instruments, Sarasota, FL). TEER values were normalized to control, uninjured cultures. The bEnd.3 Transwells were then transferred into the 12-well plates containing the astrocytes, and 3 μ M GFP or GFP-TAT was added into the top compartment of the Transwell.

After 24 h, 50 μ L of medium was collected from both the top and bottom compartments, and the fluorescence was quantified using a microtiter plate reader. The permeability (cm/sec) of GFP-TAT through the monolayer was calculated using equation 1 [7, 19]

$$P = \frac{\frac{\Delta C_B}{\Delta t} \times V_B}{C_T \times S} \quad (1)$$

$\Delta C_B/\Delta t$ is the change in fluorescence in the bottom compartment over 24 h, V_B is the volume of media in the bottom compartment (1.5mL), C_T is the fluorescence in the top compartment, and S is the surface area of the Transwell insert. The permeability of GFP-TAT through injured endothelial cultures was normalized to control, uninjured cultures.

The TEER of the monolayer was again measured, and normalized to control cultures at the same time point. The transduction of GFP into the astrocytes was quantified using the geometric mean fluorescence (excitation 488 nm, emission 530/30 nm) as measured by a FACSCanto II Flow Cytometer (Becton Dickinson, San Jose, CA) according to previously published methods [14,26]. The percentage of GFP transduction into astrocytes on the other side of the injured endothelial monolayer, compared to astrocytes in uninjured cultures, was calculated. As a positive control, GFP-TAT transduction was also measured into astrocytes without an endothelial barrier, and compared to astrocytes in uninjured endothelial-astrocyte cocultures.

Results and Discussion

Immediately following ischemic injury, the average TEER was approximately 12% lower than control cultures. Twenty-four hours after ischemia, the TEER continued to decrease, and was 20% lower than the control cultures at the same time point (Figure 1), indicating a significant decrease in the ability of the monolayer to restrict ion transport.

We have found previously that GFP-TAT permeability through the bEnd.3 monolayer was $1.2 \pm 0.2 \times 10^{-6}$ cm/s, and that this rate was comparable to similarly sized dextrans which are not transported transcellularly [27]. Our current results corroborate those results with a calculated GFP-TAT permeability of $1.0 \pm 0.1 \times 10^{-6}$ cm/s over 24 h. Following ischemia, the permeability of GFP-TAT significantly increased to $1.8 \pm 0.1 \times 10^{-6}$ cm/s. When the permeability values were normalized to the uninjured condition for each day, to account for

day to-day experimental variability, the permeability approximately doubled (Figure 2). The increased permeability after ischemic injury was comparable to the increased permeability of [¹⁴C]sucrose [21], a common marker of paracellular transport, and of 40kDa dextrans [29,31], in other *in vitro* BBB models of hypoxia. This increased GFP-TAT transport through the bEnd.3 monolayer also led to a 15% increase in TAT-mediated transduction of GFP into astrocytes cultured in the lower compartment on the other side of the bEnd.3 monolayer (Figure 3). Without the endothelial barrier, there was a $50 \pm 2.2\%$ increase in TAT-mediated GFP transduction into astrocytes compared to transduction into astrocytes in uninjured cocultures. While the injured endothelial cells still provided a barrier to the transport of GFP-TAT, the injury significantly increased paracellular transport. For the first time, we have shown that ischemia also resulted in a significant increase in TAT-mediated transduction into cells on the other side of the barrier. Our results explain why Cai *et al* and Fawell *et al* [5,13] did not observe increased TAT-mediated delivery to the brain *in vivo* through a healthy BBB while other studies reported increased *in vivo* TAT-mediated delivery in models of ischemia [6,16,17,30] and seizure [20]. This motivates further study of CPPs as brain delivery vehicles to determine the precise conditions under which TAT can deliver therapeutics into the brain parenchyma, and whether CPPs other than TAT have the ability to deliver across an intact BBB.

In this study, GFP was used as a model therapeutic cargo. Therefore, an increase in the intracellular fluorescence of astrocytes corresponded to an increase in the intracellular concentration of a protein therapeutic. Compared to DNA-based therapeutics, proteins and peptides may be highly desirable cargoes for drug delivery applications because they can be immediately active without a requirement for transcription and translation. Intracellular delivery of multiple copies of the protein cargo may be required to reach therapeutic levels within the cell. Identifying delivery systems and delivery conditions that can provide sufficient intracellular transport of a protein cargo, therefore, are essential to produce the cargo's desired therapeutic effect.

The results of this study suggest that TAT-mediated delivery may be of therapeutic benefit in situations of BBB compromise. Increased BBB permeability has been observed in inflammatory conditions such as multiple sclerosis, Alzheimer's disease and HIV-1 infection, brain tumors, traumatic brain injury, as well as hypoxia and ischemia [24]. Furthermore, increased permeability is often more substantial in regions of the BBB that are closer to the site of injury [2]. Therefore, it is likely that systemically-delivered TAT would be more likely to cross the BBB and enter the brain parenchyma closer to the injury site. As a result, TAT may be useful for targeting therapeutics to sites of injury within the brain.

Microglia and astrocytes play an important role in the brain's response to injury or inflammation. Following injury or inflammation, microglia and astrocytes can produce cytokines such as tumor necrosis factor α , interferon γ , and interleukin- 1β , which can disrupt endothelial tight junctions, thus leading to increased BBB permeability [9]. During the activation process, astrocytes increase their expression of surface glycosaminoglycans, which we have shown increases TAT transduction [26]. Therefore, activated astrocytes were transduced more readily by TAT compared to quiescent astrocytes (unpublished results). TAT's ability to permeate the disrupted BBB and transduce activated astrocytes on the other side potentially makes it an attractive delivery vehicle for the injured brain.

In this study, the permeability of GFP-TAT was monitored for 24 h after a 12 h oxygen-glucose deprivation injury. We chose this timepoint because in previous studies using bEnd.3 cells, recovery of TEER was observed 48 h following hypoxia [18]. Comparison of these results to other *in vitro* and *in vivo* studies is difficult, however, as changes in BBB permeability depend on the injury severity and reperfusion conditions [10]. *In vivo*, several

groups have reported recovery after 24 h [11,25], while other groups report persistent and widespread leakage through the BBB at 24 h [1] and 48 h [3]. Therefore, relying on BBB breakdown for delivery of therapeutics is not ideal, as delivery cannot be controlled independently and is dependent on the progression of the injury cascade.

Attempts have been made to increase BBB transport by transiently and reversibly disrupting the BBB using methods such as hyperosmotic shock, administration of vasoactive substances and focused ultrasound [8]. BBB disruption, however, has been associated with structural damage as well as the passage of plasma proteins across the BBB, altered glucose uptake, expression of heat shock proteins, microembolism, and abnormal neuronal function [23]. In addition, BBB leakage can induce astrocyte activation [15]. Therefore, there is a continued need to discover new CPPs that are capable of transporting a therapeutic across the BBB without disrupting its integrity.

Research highlights

- *In vitro* ischemia increased GFP-TAT transport across an endothelial monolayer
- Increased transport lead to increased transduction into astrocytes
- Results explain why TAT-mediated delivery has been successful in the ischemic brain
- TAT may have utility for therapeutic delivery when the BBB is disrupted.

Abbreviations

BBB	blood-brain barrier
CPP	cell penetrating peptide
GFP	green fluorescent protein
TEER	transendothelial electrical resistance

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Fig. 1.

TEER of bEnd.3 monolayers was measured immediately after 12 h oxygen glucose deprivation and again 24 h later. The data is normalized to uninjured, control cultures at each of the two time points ($n \geq 30$, mean $\pm$ SEM). The TEER of the injured cultures was significantly lower than control cultures at both time points (* $p < 0.05$ from split-plot factorial ANOVA).

Fig. 2. The permeability of GFP-TAT across bEnd.3 monolayers was significantly increased after oxygen glucose deprivation compared to uninjured, control cultures ($n > 19$, mean $\pm$ SEM; * $p < 0.05$ from Student's t-test).

Fig. 3. Oxygen glucose deprivation increased the transduction of GFP-TAT into astrocytes on the opposite side of a bEnd.3 monolayer, measured 24h post-injury ($n \geq 4$, mean $\pm$ SEM; * $p < 0.05$ from Bonferroni *post hoc* comparisons).