

**PPACK-Desmodus rotundus salivary plasminogen activator (cDSPAalpha1) prevents the passage of tissue-type plasminogen activator (rt-PA) across the blood-brain barrier and neurotoxicity.**

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**PPACK-Desmodus rotundus salivary plasminogen activator (cDSPA $\alpha$ 1) prevents the passage of tissue-type plasminogen activator (rt-PA) across the blood-brain barrier and neurotoxicity**

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Keywords:	desmotepase, blood brain barrier, tissue-type plasminogen activator, excitotoxicity

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For Peer Review

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3 **PPACK-Desmodus rotundus** salivary plasminogen activator (cDSPA $\alpha$ 1) prevents the  
4 **passage of tissue-type plasminogen activator (rt-PA) across the blood-brain barrier and**  
5 **neurotoxicity**  
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6 The treatment of ischemic stroke remains one of the most challenging areas in  
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8 medicine. To date, the only treatment approved by health authorities is early reperfusion by  
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10 the thrombolytic agent, recombinant tissue-type plasminogen activator (rt-PA)<sup>1</sup>. Nevertheless,  
11  
12 despite benefit from fibrinolysis, several limitations are linked to the use of rt-PA, including a  
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14 clinically relevant risk of haemorrhage<sup>1</sup> and a highly suspected risk of neurotoxicity<sup>2,3</sup>.  
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16 Desmoteplase (DSPA) is a highly fibrin-specific recombinant plasminogen activator (PA)  
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18 isolated from the saliva of the vampire bat *Desmodus rotundus*<sup>4</sup>. Experimental data suggest  
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20 that DSPA could display several advantages: i) in contrast to rt-PA, DSPA is devoid of pro-  
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22 excitotoxic effects both *in vitro* and *in vivo*<sup>5,6</sup>; ii) intravenous DSPA significantly reduces the  
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24 passage of co-administered rt-PA across the intact BBB and the attendant aggravation of  
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26 excitotoxic damage<sup>6</sup>. In this context, the aim of the present study was to investigate, using a  
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28 predictive *in vitro* model of BBB<sup>7</sup> (Fig. 1A), and an *in vivo* model of excitotoxicity, whether  
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30 an inactive form of DSPA, clogged DSPA (cDSPA), could prevent the ability of rt-PA to  
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32 cross the BBB and promote excitotoxic injuries. As previously demonstrated for native  
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34 DSPA<sup>6</sup>, we found that cDSPA did not alter the integrity of the BBB (passage of sucrose)  
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36 alone or in combination with rt-PA (data not shown) and can cross the BBB, despite being  
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38 catalytically inactive (Fig. 1B). Then, the passage of rt-PA alone or in combination with either  
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40 DSPA or cDSPA was analyzed using both fluorogenic proteolytic (spectrozyme®) and  
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42 zymography assays (Fig. 1C and D). Both DSPA and cDSPA reduced the passage of rt-PA by  
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44 around 30% in our *in vitro* BBB model. The effects of intravenous injection of rt-PA and  
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46 cDSPA were studied in a mouse model of excitotoxicity induced by a striatal stereotaxic  
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48 injection of NMDA (Figure 1E). Neither rt-PA nor cDSPA vehicles injected intravenously  
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50 altered the extent of NMDA-induced striatal injury. As previously demonstrated<sup>6</sup>, the  
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52 systemic injection of rt-PA increased the striatal lesion volume seen with NMDA by almost  
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3 60% (from ~10 to ~16 mm<sup>3</sup>). Interestingly, intravenous co-administration of cDSPA with rt-  
4 PA, both at 1 mg/kg, suppressed the ability of exogenous rt-PA to enhance NMDA-induced  
5 lesion. No micro-haemorrhages were detected in animals treated with rt-PA and/or clogged-  
6 desmoteplase.  
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12 Endogenous tPA, released by vascular endothelial cells, is well-known to play a  
13 critical role as a thrombolytic enzyme that activates plasminogen to plasmin. Based on this  
14 activity, exogenous rt-PA-induced thrombolysis remains the only FDA-approved drug for the  
15 treatment of patients in acute ischemic stroke<sup>1</sup>. However, t-PA favors intracerebral  
16 hemorrhagic transformation, limiting the licensed use of this drug to a 3 hours post-ictus  
17 therapeutic window<sup>1</sup>. Accordingly, the balance between the beneficial effects of thrombolysis  
18 and the injurious cerebral effects of rt-PA may be improved by restricting the access of  
19 vascular rt-PA to the brain parenchyma. Previously, we and others have demonstrated both *in*  
20 *vitro* and *in vivo* that despite its common ability to cross the intact BBB through a common  
21 transport system, in contrast to rt-PA, DSPA, is not pro-neurotoxic<sup>6</sup>. As a further  
22 characterization of the transport mechanism, our results show that a proteolytically inactive  
23 form of DSPA can restrict rt-PA trans-BBB passage. This is in agreement with the previous  
24 demonstration that PAs can cross the BBB independently of their proteolytic  
25 activity/domain<sup>8</sup>. We also demonstrate that clogged-desmoteplase prevents exogenous rt-PA-  
26 promoted excitotoxicity *in vivo*. This is in agreement with previous demonstrations that DSPA  
27 can prevent cerebral damage by rt-PA *in vivo*<sup>5,6</sup> a feature likely to contribute to the increased  
28 tolerance and safety of the clinical use of DSPA in the 3-9 hours time window post-onset of  
29 stroke symptoms<sup>9</sup>. Thus, while rt-PA remains the only approved thrombolytic drug to treat  
30 stroke patients, the interest in alternatives like DSPA is sustained. As intrinsic fibrinolytic  
31 activity of such an antagonist would not be required or might even be counterproductive,  
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3 generation of a proteolytically inactive version of DSPA able to compete with rt-PA for  
4 transport at the BBB might open the way to a safer use of rt-PA as a thrombolytic in stroke.  
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10 **Figure 1:** (A) Schematic representation of the in vitro BBB model (B) biot-rt-PA or biot-  
11 cDSPA $\alpha$ 1 were applied to the upper endothelial compartment. Two hours later, their activities  
12 or presence in the lower side medium were analyzed either by zymography assay or  
13 immunoblotting (n=3). (C,D) rt-PA alone or with DSPA or cDSPA was applied to the upper  
14 endothelial compartment. Two hours later, rt-PA activity was analyzed in the lower side  
15 medium, either by fluorogenic assay (spectrozyme®) (C) or zymography assay (D). Results  
16 are normalized to rt-PA (mean values  $\pm$  SEM of 3 experiments per group; \*  $p < 0.05$  Mann-  
17 Whitney test). (E) Effects of intravenous injection of tPA alone or in combination with  
18 cDSPA (1 mg/kg each) on the extent of neuronal death induced by the striatal administration  
19 of NMDA (10 nmol) in mice (mean  $\pm$  SD; n=10 per group; \*:  $p < 0.01$  compared to NMDA  
20 alone, \$:  $p < 0.01$  compared to NMDA + tPA, Mann-Whitney test).  
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39 **Reagents,** Human recombinant rt-PA (Actilyse®) from Boehringer Ingelheim (Paris, France).  
40 DSPA was provided by PAION Deutschland GmbH (Aachen, Germany).  
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42 **Production of cDSPA:** cDSPA was generated by coupling the Phe-Pro-Arg-  
43 chloromethylketone modified tripeptide to DSPA.  
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48 **Biotinylation:** Since cDSPA $\alpha$ 1 has no intrinsic enzymatic activity, we had to develop a  
49 method of detection for transport studies, based on biotinylation of the compound, allowing  
50 performing SDS-PAGE/immunoblotting against biotin (with a streptavidin secondary  
51 antibody). We used the FluoReporter®Biotin-XX Protein Labeling kit (Molecular Probes)  
52 according to the manufacturer's instructions.  
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3 **Blood-brain barrier (BBB) in vitro experiments:** Transport across the BBB was studied  
4 using a previously characterized in vitro model<sup>7</sup>, consisting of a co-culture of endothelial and  
5 glial cells and shown to closely mimic the in vivo BBB. rt-PA and/or desmoteplase were  
6 added to the upper side of the endothelial cells. Abluminal and luminal media were harvested  
7 after the 2 hours transport experiments.  
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14 **Spectrozyme<sup>®</sup> assays** were performed using a fluorogenic substrate (Spectrozyme XF444,  
15 American Diagnostica, Stamford, NJ, USA).  
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20 **Zymography assay** was performed by adding plasminogen (22.5µg) and casein (5mg) to a  
21 12.6% SDS polyacrylamide gel.  
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25 **Striatal excitotoxic lesions:** Mice were anaesthetised with isoflurane and placed in a  
26 stereotaxic frame. Body temperature was maintained at  $37 \pm 0.5^\circ\text{C}$ . The skull was exposed  
27 and an injection pipette was stereotaxically implanted in the right striatum according to the  
28 atlas of Paxinos & Watson (coordinates 0.5 mm posterior, +/- 2 mm lateral and -3 mm ventral  
29 to the bregma). NMDA (10 nmol) was injected in a volume of 0.3 µl. Excitotoxic treatment  
30 was followed after 10 minutes by intravenous injection of rt-PA (1 mg/kg), cDSPA (1 mg/kg),  
31 rt-PA + cDSPA (1 mg/kg each), tPA vehicle (L-Arg 35 mg/kg, phosphoric acid 10 mg/kg and  
32 polysorbate 80 0.2%) or cDSPA vehicle (glycine 4 mg/kg and mannitol 10.64 mg/kg).  
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44 **Histological analysis:** For volume analysis, one coronal section (20 µm) out of every 10 was  
45 stained with thionine and analysed with an image analyser (Scion Image, Scion Corporation,  
46 Frederick, Maryland, USA).  
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51 **Statistical analysis:** For *in vitro* and *in vivo* studies, data were expressed as mean  $\pm$  SEM, and  
52 statistical analyses were performed using the Kruskal and Wallis test followed by a Mann-  
53 Whitney test for inter-group comparisons.  
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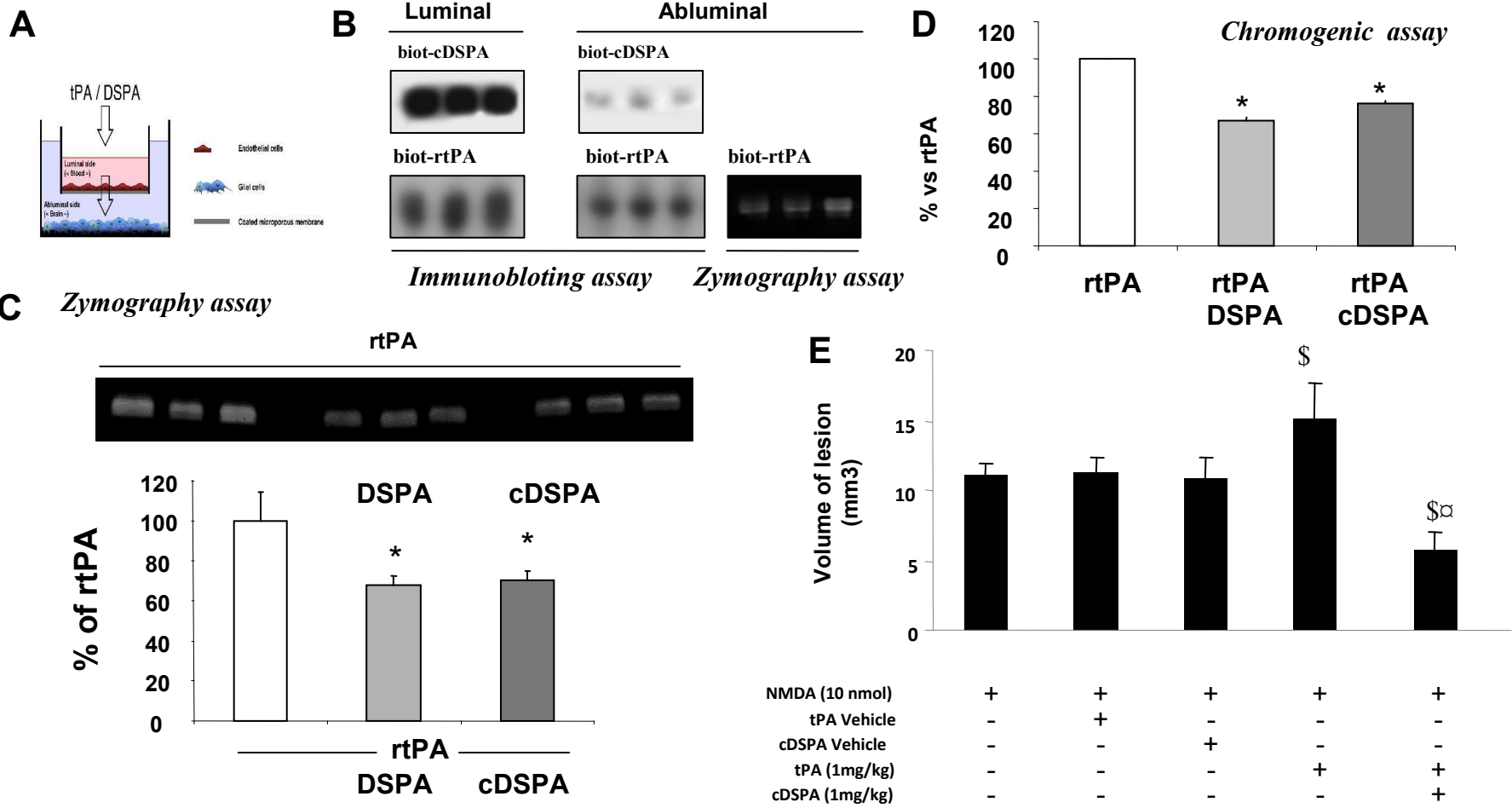
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- Based on its fibrinolytic activity, tissue type plasminogen activator (tPA) is the only treatment approved so far by the authorities, to treat ischemic stroke patients (NINDS, 1995).
  - However, exogenous tPA can display deleterious effects on components of the neurovascular unit, including promotion of edema and blood-brain barrier (BBB) leakage (Aoki et al., Stroke, 2002, Yepes et al., J.Clin. Invest., 2003), which probably minimise its overall benefit.
  - Moreover, tPA was shown to have the ability to cross both the healthy and injured BBB (Benchenane et al., Circulation, 2005), thus adding to the pro-neurotoxic effect of endogenously produced parenchymal tPA (Tsirka et al., Nature, 1995; Nicole et al., Nat. Med., 2001).
  - Desmoteplase (DSPA) is a new thrombolytic agent derived from bat salivary glands ( Hacke et al., Stroke, 2005), very close to tPA in terms of structural determinants, but previously demonstrated to :
    - cross the BBB as tPA does (Lopez-Atalaya et al., Stroke, 2007)
    - be devoid of pro-neurotoxic effect (Reddrop et al., Stroke, 2005; Lopez-Atalaya et al., J Cereb Blood Flow Metab, 2008)
  - Although the use of DSPA to treat stroke patients is debated (see DIAS 2 data), we provide here the demonstration that :
    - a proteolytically inactive form of DSPA can prevent the passage of exogenous tPA across the BBB and its subsequent neurotoxic activity, thus demonstrating the therapeutic potential of competitive inhibitors of tPA's transport to the brain for stroke patients.